University of Alberta

Mediator Expression, Storage, Mobilization and Release in Human Eosinophils

by

Salahaddin Mahmudi-Azer ©

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, the acceptance of the thesis entitled "Mediator Expression, Storage, Mobilization, and Release in Human Eosinophils" submitted by Salahaddin Mahmudi-Azer in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Medical Sciences (Medicine).

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Abstract

Eosinophils are major effector cells in allergic inflammation and asthma. A large number of eosinophil-derived mediators are stored in their unique crystalloid granules. Very little is known about mediator expression and storage, during eosinophilopoiesis, or mechanisms underlying their release from crystalloid granules of eosinophils. The tetraspanin CD63 is associated with cell activation and mediator release in basophils and neutrophils, yet its expression and association with mediator release in eosinophils remains unknown.

We hypothesized that mediators are expressed early during eosinophil granulogenesis and subsequently stored in crystalloid granules in mature cells. These mediators are selectively mobilized and released to extracellular space upon agonist stimulation. We also hypothesized that CD63 is a participant in the activation of mature eosinophil as well as selective mediator mobilization and release from crystalloid granules.

We examined mediator expression/storage and granulogenesis during eosinophil maturation in $CD34^+$ progenitor colonies. Furthermore, we examined the selective mobilization and release of eosinophil-derived RANTES, from the IFN-γ stimulated eosinophils. Finally, I studied the expression and potential association of CD63 with eosinophil mediator release.

As early as day 16 of culture of $CD34^+$projenitors expression and storage of RANTES, IL-4, MBP, and ECP were evident in maturing eosinophils. By day 28 of culture, acquisition of typical crystalloid granule-like structures, seen in circulating eosinophils, was apparent in cultured eosinophil-like cells. Upon agonist stimulation,

mediators prestored in crystalloid granules of peripheral blood eosinophils were rapidly and selectively mobilized and released to extracellular space. My studies further showed that CD63 was expressed in human eosinophils and was associated with selective mobilization and release of RANTES. This suggested that CD63 might play an important role in eosinophil activation and exocytosis. These data described here contribute to furthering our knowledge in how mediators are stored and released in human eosinophils. These findings serve as the basis for new therapeutic strategies in the treatment of eosinophil related allergic inflammatory diseases particularly asthma.

Preface

This thesis has been written in paper format according to guidelines of the University of Alberta. Each chapter stands alone as a separate document and is written in the style of the Journal of Experimental Medicine. With few exceptions, the experimental data in this thesis was generated by Salahaddin Mahmudi-Azer. In instances, where the study is the result of a collaborative work, details of the collaboration have been described.

Among various experimental procedures used in different parts of the study, eosinophil isolation and purification, inhibition of antibodies non-specific binding, cytospin preparation, immunocytochemistry and immunostaining techniques, confocal laser scanning microscopy, flow cytometry, mediator release assay, and RT-PCR were optimized and performed solely by the author of the thesis.

Acknowledgments

The last five years have been an extraordinary period of my life. I have had the pleasure and the honour of sharing my time with a wonderful group of individuals. A team of culturally and ethnically diverse individuals worked together with a powerful group dynamic. Our work environment was an academy at so many different levels and I have learned a great deal at so many fronts. Thoughts and opinions, from science to philosophy of life to culture and history were shared. Laughter often filled our faces and hearts, and each day there was something to learn and something with which to mark the passage of time. None of this would have been possible without my supervisor, Dr. Redwan Moqbel who fulfilled the roles of a great mentor and a dear friend. I would also like to acknowledge the help of Dr. Dean Befus, who kindly provided helpful insights and shared his views.

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Abbreviations

Adenosine Triphosphate, Aprotinin, Nα-*p*-tosyl-L-arginine Methyl Ester (TAME)

Antibody (Ab)

Antigen-Presenting Cells (APCs)

Base Pair (bp)

β-hexosaminidase (β-hex)

Bronchoalveolar Lavage (BAL)

Bronchial Hyperresponsiveness (BHR)

Botulinum Toxin (BoNT)

Bovine Serum Albumin (BSA)

Charcot-Leyden Crystals (CLC)

Cluster Designation (CD)

Colony Forming Unit-Granulocyte/Erythroid/Macrophage/Megakaryocyte (CFU-GEMM)

Confocal Laser Scanning Microscopy (CLSM)

Cytotoxic T-Lymphocyte (CTL)

Cytochalasin B (CB)

Differential Interference Contrast (DIC)

Extracellular Matrix (ECM)

Eosinophil/Basophil-Colony Forming Unit (Eo/B-CFU)

Eosinophil-Derived Neurotoxin (EDN)

Eosinophil Cationic Protein (ECP)

Eosinophil Peroxidase (EPO)

Fetal Calf Serum (FCS)

5-lipoxygenase (5-LO)

5- lipoxygenase -activating protein (FLAP)

Fluorescence-Activated Cell Sorter (FACS)

Fluorescein Isothiocyanate (FITC)

Gravity (g)

Granulocyte-Colony Stimulating Factor (G-CSF)

Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF)

Granulocyte/Macrophage-Colony Forming Unit (GM-CFU)

Macrophage-Colony Stimulating Factor (M-CSF)

N-ethylmaleimide Sensitive Factor (NSF)

Hemopoietic Stem Cell (HSC)

Heparin-Binding Epidermal Growth Factor (HB-EGF)

Immunoglobulin G (IgG)

Immunocytochemistry (ICC)

Interleukin (IL-)

In Situ Hybridization (ISH)

Interferon-γ (IFNγ)

Isoelectric Point (pI)

Kilo Dalton (KDa)

Lactate Dehydrogenase (LDH)

Leukocyte Function Antigen (LFA-1)

Leukotriene C_4 (LTC_4)

Leukotriene B_4 (LTB_4)

Lipopolysaccharide (LPS)

Litre (L)

Lipoxin-A_4 (LXA_4)

Lymphotoxin (LT)

Lysosome-Associated Membrane Protein-3 (lamp3)

Major Histocompatibility Complex (MHC)

Major Basic Protein (MBP)

Mean Fluorescent Intensity (MFI)

Milligram (mg)

Milliliter (mL)

Millimolar (mmol)

Microgram (μg)

Messenger Ribonucleic Acid (mRNA)

Macrophage Inflammatory Peptide-1 alpha (MIP-1α)

Myeloperoxidase (MPO)

Nerve Growth Factor (NGF)

NSF (N-Ethylmaleimide Sensitive Factor)

Phosphate-Buffered Saline (PBS)

Piecemeal Degranulation (PMD)

Platelet-Activating Factor (PAF)

Prostaglandin-E_2 (PGE_2)

Phenylmethylsulfonyl Fluoride (PMSF)

Rapidly Releasable Pool (RRP)

Rat Basophilic Leukemia Cell Line (RBL)

Regulated upon Activation Normal T-Cell Expressed and Secreted (RANTES)

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Stem Cell Factor (SCF)

Soluble NSF Attachment Protein (SNAP)

SNAP Receptor (SNARE)

Synaptosome-Associated Protein of 25 kD (SNAP-25)

Tetramethylbenzidine (TMB)

Tetanus Toxins (TeNT)

Transforming Growth Factor α/β (TGFα/β)

Thromboxane-B_2 (TXB_2)

Tumor Necrosis Factor (TNF)

Tris-Buffered Saline (TBS)

Transmembrane-4 Superfamily (TM4SF)

Target SNARE (t-SNARE)

Vesicle Associated Membrane Protein-1 (VAMP-1)

Vesicular SNARE (v-SNARE)

Chapter 1

General Introduction

Asthma, Eosinophils and Mediator Release

Asthma

1. Introduction

The term 'asthma' describes a heterogeneous collection of clinical manifestations and characteristics, as opposed to a single condition. Asthma results from complex interactions between wide ranges of environmental factors acting on a background of genetic predisposition. Although, at the beginning of the 20th century asthma was a rare disease, almost a hundred years later it is approaching a true epidemic in developed countries. While millions of people are affected worldwide by asthma, attempts to define this disease were not successful until very recently. In the early 1950s, asthma definition emphasized on the reversibility of airflow obstruction, either spontaneously or as a result of treatment (1). In the early 1960s, the presence of bronchial hyperresponsiveness (BHR) was added to the definition of asthma (2).

In the mid 1980s, bronchoscopic examinations of asthmatic patients led to a new era in understanding and defining asthma (3). By employing this procedure, scientists in the field were able to carry out close and detailed examination of the airways. This close examination of the asthmatic airways revealed the presence of an intense inflammatory response characterized by the presence of eosinophils and mucosal sloughing in patients with newly diagnosed asthma who had a mild form of the disease (4).

As a result of decades of intense studies, asthma is now defined at least in Canada as "a chronic inflammatory disease of the airways in which many cell types play a role, in particular, mast cells, eosinophils and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli" (5, 6). Current evidence indicates that airway inflammation plays a central role in the pathogenesis of asthma and it is a driving force in airway hyperresponsiveness and the susceptibility to airflow obstruction (7). It is unknown whether inflammation is the only cause of airway hyperresponsiveness; nevertheless it is clear that inflammation is closely associated with the development of airway hyperresponsiveness. It has been proposed that the intensity of airway inflammation not only contributes to the degree of airflow limitation but also is a major factor in determining the level of airway hyperresponsiveness (8).

2. Asthma as an inflammatory disease

Asthmatic inflammation develops as a result of complex interactions among a wide variety of inflammatory cell types and their respective mediators (9). Infiltration of inflammatory cells into the airway walls is a fundamental feature of asthma (10). Although, asthma is characterized by a specific form of bronchial inflammation mainly mediated by activated T cells (11), eosinophils (12) and mast cells (13), it is becoming increasingly apparent that other cell types including epithelial cells (14), fibroblasts (15), smooth muscle cells(16) and neutrophils (17) may also contribute to the inflammatory events (fig. 1).

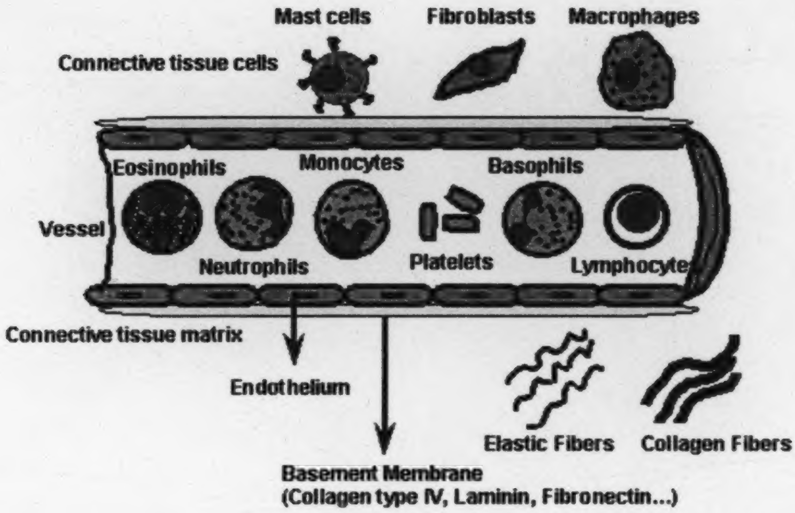

Mast cells
Fibroblasts
Macrophages
Connective tissue cells
Eosinophils
Monocytes
Basophils
Vessel
Neutrophils
Platelets
Lymphocytes
Connective tissue matrix
Endothelium
Elastic Fibers
Collagen Fibers
Basement Membrane
(Collagen type IV, Laminin, Fibronectin...)

Figure1.1: Intravascular cells and connective tissue matrix proteins and cells involved in inflammatory responses.

T cells, central cells in the adaptive immune response, are thought to coordinate and amplify the effector functions of antigen-specific and nonspecific inflammatory cells such as B cells, mast cells and eosinophils (18, 19). T cells, which express $\alpha\beta$ receptors can be divided into two major functional groups according to their expression of the phenotypic surface molecule CD4 and CD8. The $CD4^+$ T cells also known as T-helper cells (Th), are now recognized as the important players in inflammatory response, whereas $CD8^+$ T cells, termed cytotoxic T lymphocytes (CTL), largely function to eliminate body cells expressing new (non-self) surface antigens as a result of bacterial or viral infection or malignant transformation. The $CD4^+$ T cells are particularly important in the regulation of antigen-driven inflammatory processes, but this does not exclude the possibility of a role for the $CD8^+$ T cell (20,21).

On the basis of murine studies on cloned T-cells, T-helper cells responses are classified into 2 subtypes, depending on their cytokine profile in response to antigenic stimulation. Th1 cells, with a cytokine profile of IFN-γ, IL-2, IL-12, IL-18, TNF and lymphotoxin (LT), which are important in activating macrophages and inducing cell-mediated immunity, and Th2 cells with cytokine profile of IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 together with IL-3 and GM-CSF (shared by both the Th1- and Th2-type T cells) which are important in humoral immunity and allergic inflammatory conditions (22, 23). A sequence of differentiation steps driven by exposure to antigen are thought to give rise to Th1 and Th2 cells from common precursor cells, which secrete only IL-2 (24). $CD4^+$ T cells first differentiate into Th0 cells, which are known to have an unrestricted cytokine profile. Following continuous and chronic antigen stimulation, the $CD4^+$ T cells subsequently differentiate into polarized Th1 or Th2 cells (25, 26) (fig.2).

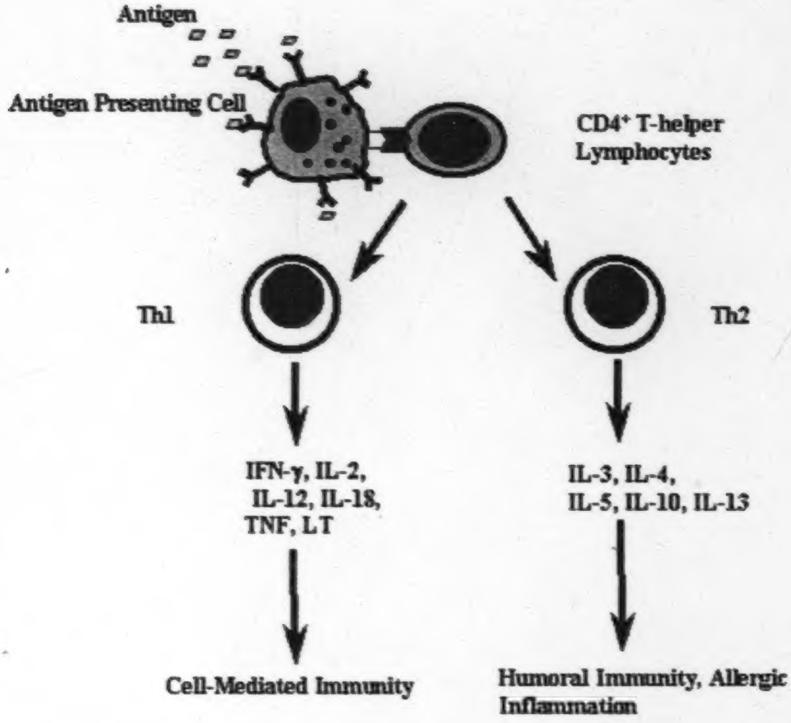

Antigen
Antigen Presenting Cell
CD4⁺ T-helper
Lymphocytes
Th1
Th2
IFN-γ, IL-2,
IL-12, IL-18,
TNF, LT
IL-3, IL-4,
IL-5, IL-10, IL-13
Cell-Mediated Immunity
Humoral Immunity, Allergic
Inflammation

Fig. 1.2: CD4$^+$ T cells differentiation into polarized Th1- or Th2- type cells.

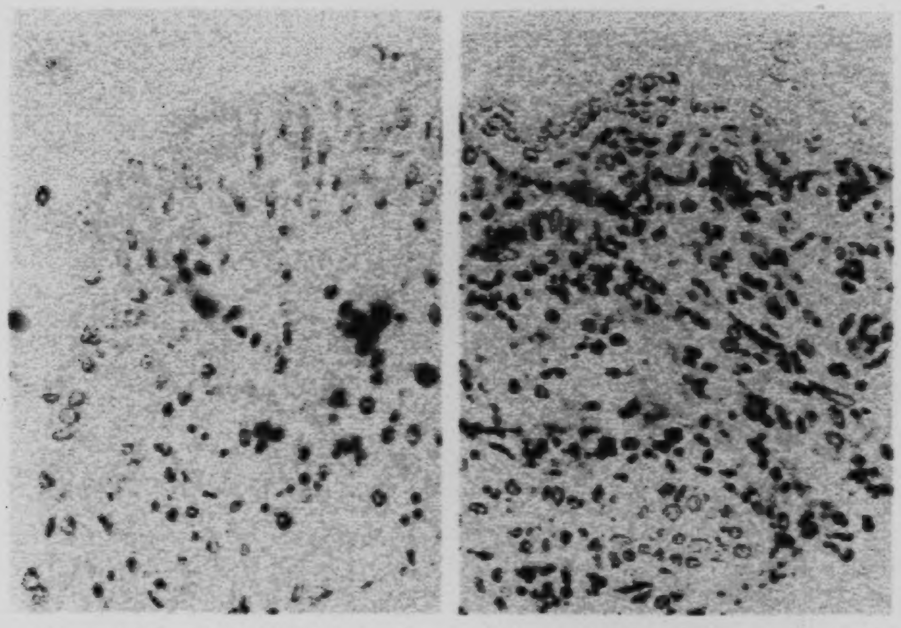
The specific cytokine profile that develops in CD4$^+$ T cells in both allergic and nonallergic diseases is not predetermined. Instead, it is influenced by the antigen type, dose, antigen-presenting cell type, cytokine microenvironment, costimulatory signals received by the T cell, and host or genetic factors (27-31). Th1 cytokines inhibit the production of Th2 cytokines, whereas Th2 cytokines inhibit the production of Th1 cytokines, thus emphasizing the production of polarized cytokine profiles during immune responses. Once CD4$^+$ T cells fully differentiate into effector Th1 and Th2 cells, the cytokine profiles become relatively fixed. However, Th1 and Th2 cells, with discrete cytokine profiles and capacities to respond to IL-12 and IFN-γ, represent ends of a phenotypic spectrum of Th cells. Other types of effector T cells, producing a variety of other combinations of cytokines, such as IL-4 with IFN-γ, can be observed in other situations (32,33).

The premise of Th1 and Th2 and the overall heterogeneity among CD4$^+$ T cells has become widely accepted since it explains the inverse relationship between humoral and cell-mediated immunity, which has been observed clinically and experimentally for decades. CD4$^+$ T cell heterogeneity also explains the development of allergic inflammatory disease as the result of an excessive production of Th2 cytokines (IL-3, IL-4, IL-5, IL-10, and IL-13) by allergen-specific CD4$^+$ Th2 cells. Indeed, it is now widely accepted that Th2 cells assume a prominent role in orchestrating allergic inflammatory responses. Upon synthesis and release, Th2 -type cytokines are known to induce the infiltration of inflammatory cells into the airway walls (predominantly eosinophils and lymphocytes). A sequential interaction of these cells with resident cells (e.g., mast cells, macrophages, epithelial cells, endothelial cell etc.) generates a cascade of events that contributes to the chronic inflammation and clinical manifestations associated with asthma. Mediators, generated by these interactions attract other inflammatory cells, thereby contributing to further inflammation, airway smooth muscle spasm (bronchospasm), airway mucus secretion, and airway microvascular leakage that lead to airway edema and narrowing. (34,35).

Although, the Th1 and Th2 model seems to simply explain certain aspects of immune regulation and function, a number of observations suggest that immune regulation is far more complex than initially suggested by the Th1/Th2 model. For example, Th2 cells, identified mainly by the capacity to produce IL-4 but not IFN-γ, may be further subdivided. The amount of cytokines produced by Th2 cells (e.g., IL-5, IL-10, or Lymphotoxin) may vary considerably and change the specific functions of Th2 cells (36).

3. Pathogenesis of asthma

Bronchial hyperresponsiveness is an important pathophysiological characteristic of asthma, which can describe many of the clinical features of this disease (37). Bronchial hyperresponsiveness has been defined as an abnormal increase in airflow limitation following the exposure to stimulus. Almost all adult patients with symptomatic asthma have airway hyperresponsiveness to inhaled pharmacological stimuli (38). Bronchial hyperresponsiveness is thought by many to be closely associated with the infiltration and accumulation of inflammatory cells and consequent inflammatory response in the airways (39) (fig. 3). In addition to recruitment of mature cells from the blood stream, tissue infiltration of inflammatory cells may also involve progenitors of inflammatory cells, which further proliferate in the submucosal tissue (40,41,42).



Eosinophil recruitment in the asthmatic lung

Airway of Resting Mild Asthmatic

Airway of Same Subject, 6 h Post Allergen Challenge

Fig. 1.3: Recruitment of eosinophils into the airways of an asthmatic patient. Airway biopsy of an asthmatic individual before and after allergen challenge was immunostained with specific antibody against eosinophil cationic protein (ECP). As shown, ECP^+ immunostaining is increased following allergen challenge suggesting eosinophil recruitment into the airways (copied with permission from an original figure from Dr. Redwan Moqbel).

In addition to inflammatory cells, structural tissue cells including endothelial and epithelial cells (43,44), fibroblasts (45,46), and smooth muscle cells (47,48) can also play an important regulatory role in inflammation, through the release of a variety of cytokines, chemokines and growth factors. Infiltration of inflammatory cells and their activation together with that of structural tissue cells at the site of inflammation result in an acute local response characterized by vascular leakage, mucus hypersecretion, epithelial shedding, and widespread airway narrowing. Furthermore, through the release of proinflammatory mediators such as histamine, platelet-activating factor (PAF), derivatives of the arachidonic acid cascade, such as prostaglandins and the cysteinyl leukotrienes, and toxic oxygen radicals, these cells cause persistence of the inflammatory infiltrate and induce structural changes in the airway wall known as tissue remodeling (49,50).

Components of the extracellular matrix (ECM) have also been shown to influence the inflammatory response profoundly (51,52). In addition to containing released mediators, ECM proteins also act as ligands for cell adhesion molecules on inflammatory cells with the potential to effect infiltration and trafficking of cells (53,54). ECM proteins can also activate granulocytes, increase mediator release and enhance their survival (55,56). Equally, several inflammatory mediators are known to affect the proliferation of airway smooth muscle and induce the synthesis of ECM proteins and growth factors in fibroblasts and epithelial cells (57).

Emphasis on reversibility of airflow obstruction as a defining feature of asthma may have delayed the recognition of its potential to cause irreversible airflow obstruction. For decades, asthma was considered a completely reversible obstructive airway disease

(58,59). This was in spite of many patients showing evidence of some residual airway obstruction. Several studies of asthma in older adults gave rise to the idea that asthma can lead to irreversible airflow obstruction. This irreversible component is more prominent in patients with severe disease and even may persists after an aggressive course of anti-inflammatory treatment (60,61).

The process responsible for the permanent narrowing of the airway lumen is a chronic, irreversible thickening of the walls of the airways described as "airway wall remodeling" (62,63). Thickening of the airway wall has been demonstrated by pathologic studies and by radiographic studies using high-resolution computed tomography (64,65). The pathologic changes responsible for airway wall thickening include infiltration of the airway wall by inflammatory cells, edema, hyperplasia of epithelial goblet cells and of mucus-secreting glands, subepithelial collagen deposition, subepithelial accumulation of myofibroblasts, and thickening of the medial smooth muscle layers (66,67). These pathologic changes are presumed to result from repeated or chronic release of the cytokines, proteases, and other chemical mediators of inflammation (68).

4. Development of atopy and asthma

It is believed that individuals with allergic asthma exhibit a cellular reaction in response to exposure to specific antigen(s) (69). The current model proposes that the first exposure to the antigen (sensitization) triggers the potential for these responses particularly in individuals with certain major histocompatibility complex (MHC) alleles and other genetic predispositions (70, 71). Upon exposure to a novel antigen and its contact with airway mucus, underlying naive airway epithelial cells and resident dendritic cells are primed. It is also likely that the antigen will penetrate the underlying submucosa, where it may come in contact with phagocytes (e.g., tissue macrophages, granulocytes) and enter lymphatics (72). It is believed that all of these cells can internalize, process, and present fragments of the antigen in association with MHC of either class. Antigen-presenting cells (APCs) play a major role in activating lymphocytes responsible for setting the stage for subsequent local atopic responses (73). During primary exposure several cellular players are recruited, and primed. Upon a second exposure to the same antigen, primed cells respond through the release of proinflammatory mediators, which are directly responsible for rapid (within minutes) inflammatory response characterized by wheezing and reduced airflow. In many individuals with atopic asthma, depending on the dose of allergen exposure, a clinical late-phase response (3 to 11 hours) will follow the early phase response, consisting principally of a second delayed-in-time of bronchoconstriction. The late-phase response represents a longer-term outcome of cellular activities that were set in motion during the early phase (74, 75).

5. Early phase response

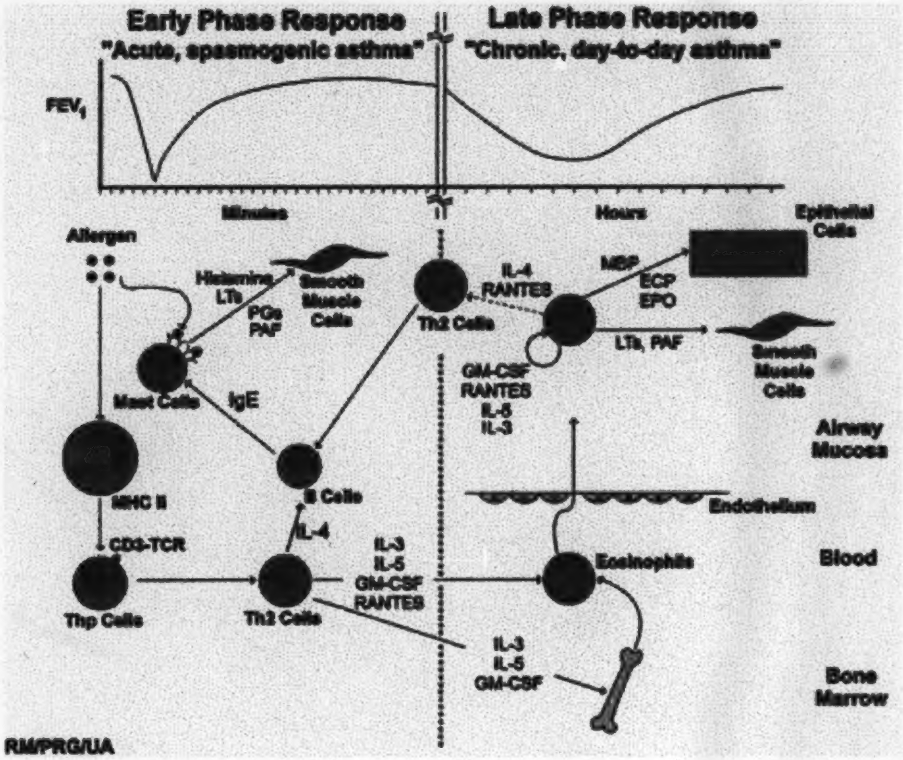
Inhaled allergen challenge in previously sensitized allergic patients leads to an early (immediate) allergic inflammatory reaction. The early-phase response is initiated after the activation of cells bearing allergen-specific IgE receptor. This is characterized by rapid activation of airway mast cells and macrophages (76, 77). Cell types other than basophils and mast cells that bear the high-affinity IgE receptor (FcεRI) may also participate in early-phase response, but it is not known whether they can be activated directly by allergens (78). The observation that mast cell-derived mediators can initiate many typical characteristics of early-phase response such as vascular, bronchial, and cellular changes, indicate a central role for airway mast cells in early phase response.

Activated mast cells rapidly release a number of proinflammatory mediators such as histamine, leukotriene C_4 (LTC_4), PAF, and bradykinin, which are known to increase vascular permeability leading to airway edema and clinical symptoms of congestion and in some cases rhinorhea. Several mast cell products including histamine, LTC_4, and prostaglandin D_2 (PGD_2) are smooth muscle constrictors and may induce bronchoconstriction. Increased mucus secretion is attributed to leukotrienes and possibly chymase (79-82) (fig. 4).

6. Late-phase response

Of particular importance to the development of a clinical late-phase response is the recruitment of other inflammatory cells to the site of inflammation. Some mast cell-derived mediators are chemoattractants (leukotriene B_4, PAF) that stimulate the influx of inflammatory and immune cells several hours after allergen exposure (83,84).

Hours after allergen challenge, large number of eosinophils, lymphocytes neutrophils, as well as some basophils are recruited to airway tissues including submucosa, epithelium, and airway lumen (85,86). A large number of cellular adhesion molecules are known to be involved in selective recruitment of the inflammatory cells (87,88). Expression of some of the endothelial adhesion molecules (VCAM-1, ICAM-1, and E-selectin) are upregulated by certain cytokines, including TNF and IL-4, which are released during the early phase, suggesting that endothelial changes enhance late-phase leukocyte infiltration of airway tissues (89,90). In spite of extensive data about the existence of endothelial cell/leukocyte adhesion molecule pairs, the extent to which their expression has an impact on the course of asthma and allergic rhinitis awaits confirmation (91,92). Currently, expression of VCAM-1 appears to be upregulated in those with allergic inflammation after allergen challenge (93). The accumulation of eosinophils in airway tissue and the release of their mediators are known to be a prominent component of the late-phase response. Eosinophils, like mast cells, appear to generate a variety of pro-inflammatory mediators as well as cytolytic enzymes that disrupt airway epithelial cell integrity (94,95) (fig. 4).



Early Phase Response
"Acute, spasmogenic asthma"
Late Phase Response
"Chronic, day-to-day asthma"
FEV_1
Minutes
Hours
Allergen
Histamine
LTs
PGs
PAF
Smooth Muscle Cells
Mast Cells
IgE
MHC II
CD3-TCR
Thp Cells
Th2 Cells
IL-4
B Cells
IL-3
IL-5
GM-CSF
RANTES
Th2 Cells
IL-4
RANTES
MBP
ECP
EPO
Epithelial Cells
LTs, PAF
Smooth Muscle Cells
GM-CSF
RANTES
IL-5
IL-3
Airway Mucosa
Endothelium
Eosinophils
Blood
IL-3
IL-5
GM-CSF
Bone Marrow
RM/PRG/UA

Fig. 1.4: The current paradigm of the pathogenesis of both early and late phase asthmatic responses (copied with permission from an original figure by Dr. Redwan Moqbel).

II. Eosinophils

1. Introduction and historical perspective

Using a compound microscope and the blood of several animals, the English anatomist, T. W. Jones, in 1846 discovered that some white blood cells contained granular structures that became visible when immersed in hypotonic solutions (96). Although, it has been claimed that Jones had discovered the eosinophil (97), more likely he visualized the more abundant neutrophil (98). In the latter decades of the 19th century, perhaps Brown was the first to detect eosinophils in the blood and bone marrow of patients with eosinophilic leukemia yet, the lack of appropriate dyes and staining techniques at that time prevented formal identification (99). About 120 years ago Paul Ehrlich (100) noticed that a certain population of white blood cells stained with a negatively charged, brominated fluorescein compound, eosin. Therefore, he named this population of white blood cells "eosinophil". Decades later, Ehrlich received full credit for the discovery of the eosinophil.

During the next several decades, much was learnt about the biology of eosinophils including their critical role in host defense and allergic diseases (101,102). Elevated numbers of eosinophils were associated with atopic dermatitis (103), allergic rhinitis (104), asthma (105), and helminth infections (106). The number of eosinophils was also markedly increased in animal tissues after acute anaphylaxis (107). The role of the eosinophil in anaphylaxis remained the dominant explanation for eosinophil function from the early part of 20th century until the 1980s. It is now recognized that the eosinophils synthesize, store and secrete a number of cytotoxic mediators able to kill helminths (at least *in vitro*), damage mucosal tissues and cause disease.

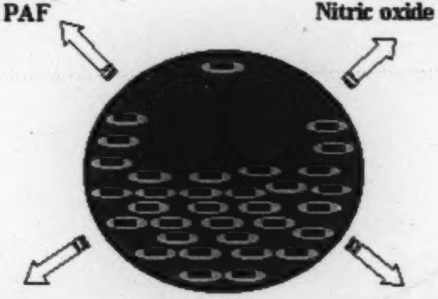
2. Eosinophil morphology and ultrastructure

Eosinophils are end differentiated leukocytes derived from the bone marrow, and have been identified in many mammalian and non-mammalian species. Human eosinophils are approximately 10-15 μm in diameter, with bilobed or trilobed nuclei and several distinct characteristics that distinguish them from other granulocytes (108,109). The synthetic structures of eosinophils (Golgi apparatus and endoplasmic reticulum) are markedly reduced over their immature precursors. A prominent feature of the eosinophil is the presence of a large number of granular structures that occupy approximately one-fifth of the cytoplasm. In addition to nonmembrane-bound lipid bodies, cytoplasmic secretory granules of at least three types (secondary granules, small granules and primary granules) have been characterized in eosinophils (110,111). Eosinophil secretory granules provide a major means for the ultrastructural identification of eosinophils. Indeed, the first morphological marker of the eosinophil is the appearance of granules that are visible at the promyelocyte stage (112). Further, the emergence of crystalloid core-containing secondary granules in small numbers in immature precursors allows the assignment of such cells to the eosinophil lineage (113) (fig. 5).

Eosinophil Mediators

De novo **synthesized lipid mediators: LTC_4, PGE_1, PGE_2, TXB_2, 15-HETE, PAF**

Oxidative metabolites
Reactive oxygen species
Nitric oxide



Preformed granule derived cationic proteins
Major basic protein (MBP)
Eosinophil peroxidase (EPO)
Eosinophil cationic protein (ECP),
Eosinophil-derived neurotoxin (EDN),
Charcot-Leyden crystal protein (CLC)

Cytokines, chemokines and growth factors
IL-1α, IL-2, IL-3, IL-4,
IL-5, IL-6, IL-8, IL-9,
IL-10, IL-12, IL-16,
TGF α, TGF-β, SCF, NGF,
IFN-γ, TNF, GM-CSF,
RANTES, Eotaxin, MIP-1α

Fig. 1.5: Eosinophil-derived mediators. (Modified with permission from a schematic design by Dr. Redwan Moqbel)

3. Eosinophil granules

A. Specific secondary (crystalloid) Granules

In human peripheral blood eosinophils, specific granules are bicompartmental, membrane-bound, spherical structures with a centrally located crystalline core compartment surrounded by an outer matrix compartment (110). Using immunogold staining, a number of preformed proinflammatory cytokines/chemokines and cationic eosinophil mediators have been localized to the specific granules. Major basic protein (MBP), a cytotoxic protein known to damage a wide variety of mammalian cells (114,115) and parasites (116-118), is predominantly present in the core compartment (119); while, eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) reside in the matrix compartment (119,120). Specific granules, containing multiple cores, have also been visualized (121), but their occurrence is relatively rare (fig 6).

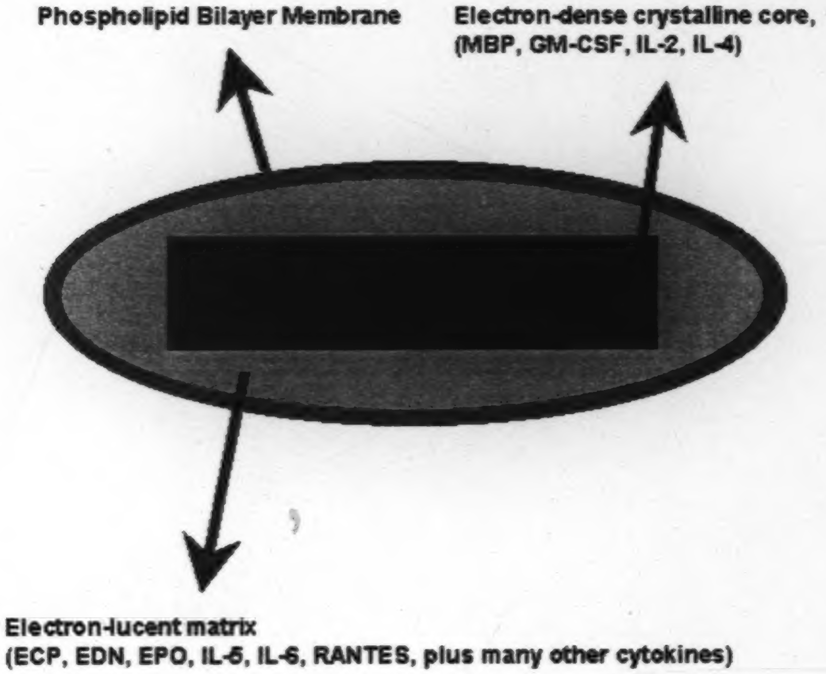

Phospholipid Bilayer Membrane
Electron-dense crystalline core, (MBP, GM-CSF, IL-2, IL-4)
Electron-lucent matrix (ECP, EDN, EPO, IL-5, IL-6, RANTES, plus many other cytokines)

Fig. 1.6: The specific (crystalloid) granule of eosinophils. (Modified with permission from a schematic design by Dr. Redwan Moqbel)

B. Primary Granules

Primary granules are roughly spherical, of variable size, and contain no discernable core. They account for approximately 5% of all eosinophil granules. In resting eosinophils, primary granules provide the sole location for the Galectin- 10 (Charcot-Leyden Crystals CLC) (122). These are colorless and have a characteristic hexagonal, bipyramidal structure with intrinsic lysophospholipase activity (123). Although, CLC protein possesses lysophospholipase activity, its role(s) in eosinophil or basophil function or associated inflammatory responses has remained speculative. The overall structural fold of CLC protein is highly similar to that of galectins -1 and -2, members of an animal lectin family formerly classified as S-type or S-Lac (soluble lactose-binding) lectins. In activated cells, trace amounts of CLC have been identified within the nucleus and cytoplasm, implying that this protein can be released intracellularly (124).

C. Small Granules

These small structures (<0.5 μm) have been identified in human tissue eosinophils, but are not seen within circulating cells or those in the bone marrow (125). They have been shown to contain arylsulphatase-B and acid phosphatase by cytochemical studies (125), and only one immunogold analysis has localized catalase to these granules (126). Thus, their function may be similar to that of lysosomes and peroxisomes in other cells.

D. Lipid bodies

Lipid bodies represent specialized intracellular domains that form rapidly in response to agents that activate protein kinase C. Lipid bodies are large, non membrane-bound structures of lipid that are present in small numbers (approximately five per cell) in mature eosinophils. Their number increases in activated cells (127). Lipid bodies are spherical (0.5-2 μm in diameter), electron-dense organelles that provide a principle site of storage for arachidonic acid (AA), which is esterified into glycerophospholipids (128). Furthermore, lipid bodies serve as sites at which the coordinated and regulated enzymatic events involved in arachidonate mobilization and oxidative metabolism could occur.

Eosinophils and Asthma

There is a substantial body of evidence indicating the recruitment of eosinophils into the airways of asthmatic individuals, which is regulated by T-cell-derived products (fig. 3). The infiltration of the airways with eosinophils has been shown to occur in mild atopic as well as non-atopic asthma (129,130). A large numbers of eosinophils have been shown to surround the bronchi in patients who have died of asthma (131,132). Eosinophil-derived granule cationic proteins have been detected in bronchoalveolar lavage (BAL) fluid of both symptomatic and asymptomatic asthmatic subjects (132,133).

The increased presence of eosinophils within asthmatic lungs and their activation has been associated with allergen induced early and late phase response as well as occupational asthma to various inciting agents (134-136). Within the airways of asthmatics, eosinophils are believed to release a number of proinflammatory granular cationic proteins including MBP, ECP, EPO and EDN. These cationic proteins have been associated with some of the pathophysiologic aspects of asthma, including cellular cytotoxicity, mast cell and basophil degranulation, and mucus hypersecretion (137). Although, elevated levels of each of the four cationic proteins can be detected in the bronchoalveolar lavage fluid of asthmatic individuals, only MBP has been shown capable of provoking both bronchoconstriction and airway hyperreactivity in primates (138). The cytotoxic properties of MBP toward respiratory epithelium (139), together with localization of MBP at the sites of bronchial damage (140), led to a widely held belief that the cytotoxicity is responsible for the pathogenic consequences of MBP in asthma (fig. 7).

Although a large number of studies have highlighted a critical role for eosinophils in airway hyperresponsiveness and the late asthmatic response, a recent study which examined the effects of a humanized IL-5 blocking monoclonal antibody on eosinophil numbers, airway hyperresponsiveness, and the late asthmatic response, questioned the role of eosinophils in the asthmatic response (141). In this study the effects of anti-IL-5 on blood and sputum eosinophils, airway hyperresponsiveness, and the late asthmatic reaction to inhaled allergen in patients with mild asthma have been assessed. The authors suggested that while anti-IL-5 induced a significant reduction in the sputum and peripheral blood eosinophilia, it did not produce significant effect of the late asthmatic response or airway hyperresponsiveness to histamine. This controversial study is interesting but has a number of flaws that prevents unambiguous conclusions. Particularly the study was underpowered by sample size, and the patient selection was very restrictive. Finally, the study confined the investigation to effects of anti-IL-5 antibody to peripheral blood and sputum eosinophils, without looking at tissue eosinophils. Further and better-designed studies are needed to elucidate the role of eosinophils in asthma. Even though eosinophils may have a wide spectrum of functions, it might be difficult to conclude their role in asthma.

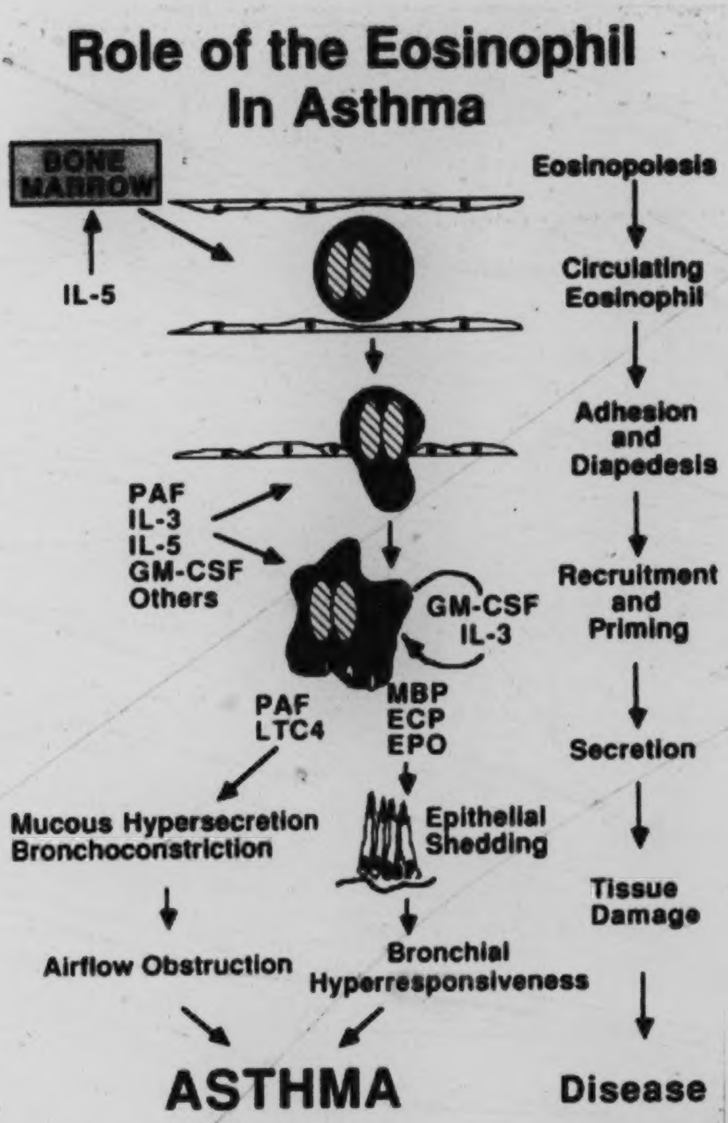

Role of the Eosinophil
In Asthma
BONE MARROW
IL-5
Eosinopoiesis
Circulating Eosinophil
Adhesion and Diapedesis
PAF
IL-3
IL-5
GM-CSF
Others
GM-CSF
IL-3
Recruitment and Priming
PAF
LTC4
MBP
ECP
EPO
Secretion
Mucous Hypersecretion
Bronchoconstriction
Epithelial Shedding
Tissue Damage
Airflow Obstruction
Bronchial Hyperresponsiveness
ASTHMA
Disease

Fig. 1.7: Role of eosinophils in the pathogenesis of asthma (copied with permission from an original figure by Dr. Redwan Moqbel & Andrew J. Wardlaw).

III. Eosinophil Growth and Differentiation

Introduction

Eosinophils are specialized proinflammatory cells that arise in the bone marrow and traffic via the blood to various mucosal sites where they are believed to exert their effector functions. Selective activation of eosinophil-differentiation pathways in the bone marrow may be an important event in the development and maintenance of allergic inflammatory responses. A large number of studies, which have tried to elucidate the underlying mechanisms and pathways leading to eosinophil differentiation, have provided further means for therapeutic interventions in allergic inflammation.

Hemopoietic stem cells (HSCs) are the earliest recognizable inflammatory cell precursor in the bone marrow. HSCs are defined by their capacity for long-term self-renewal and differentiation along multiple lineage pathways (142). The development of mature hemopoietic cells from these pluripotent HSCs involves a regulated progression through lineage commitment, terminal differentiation and growth arrest (143). Cytokines/growth factors, as well as exogenous stimulation have been shown to be associated with the self-renewal and lineage commitment of HSCs. It has been shown that a controlled proliferation of primitive cells follows the fate determining stochastic mechanisms (144-147). A commonly used marker in identifying and isolating the progenitors is the CD34 antigen. CD34 is a monomeric, transmembrane glycophosphoprotein, whose expression within the hemopoietic system is restricted to early lymphohemopoietic stem cells and progenitors (148-149). CD34 may play an important role in progenitor cell adhesion and activation within the bone marrow stromal compartment (150).

Myelopoiesis

Eosinophils and neutrophils are derived from a common myeloid progenitor lineage known as the CFU-GEMM (colony forming unit-granulocyte/erythroid/macrophage/megakaryocyte), which can further differentiate to GM-CFU (Granulocyte/Macrophage-colony forming unit) and Eo/B-CFU (Eosinophil/Basophil-colony forming unit). These two progenitor types are the earliest recognizable progenitors that can be identified using semisolid culture techniques. At least four different cytokines are known to induce the differentiation of GM-CFU into mature neutrophils, macrophages or both. These include IL-3, GM-CSF, G-CSF and M-CSF (151,152).

Eosinophilopoiesis

Eosinophil turnover, or eosinophilopoiesis, occurs almost exclusively in the bone marrow although additional sites of production can include the spleen, thymus, and lymph nodes. The ultimate commitment of the stem cells to unipotential progenitors, and their subsequent survival and expansion into the mature eosinophils, has been studied extensively. However, a complete understanding of the factors and processes by which this occurs is still lacking. The eosinophil and basophil share a common late stage progenitor (Eo/B CFU), evidenced by shared content of the major granule proteins and proteoglycans (153-157), and the occurrence of various forms of transition between the two cell types (153-155, 158). IL-3, IL-5 and GM-CSF have been shown to regulate the commitment to the eosinophil and basophil lineages (159,160). IL-5, which acts on a late-stage progenitor, specifically supports the terminal differentiation of the eosinophil and basophil (161,162). While there is some evidence that IL-3 alone may support differentiation along the basophil lineage, this cytokine has not been shown to be as specific as IL-5 (163). Several growth factors such as G-CSF and stem cell factor (SCF), that are not specific for eosinophil lineage, are thought to act on early eosinophil progenitors in a synergistic fashion (164,165). IL-3, but not IL-5, can induce colony formation from CD34$^+$ progenitors. However, only when these cells are grown first in IL-3 followed by IL-5, do eosinophil colonies emerge (164) (fig. 8).

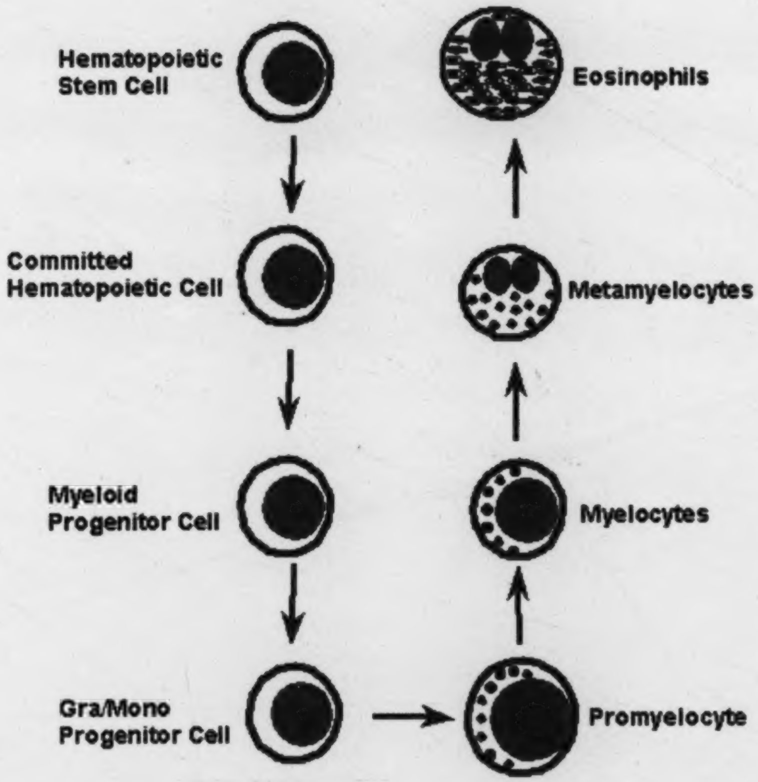

Hematopoietic Stem Cell
Committed Hematopoietic Cell
Myeloid Progenitor Cell
Gra/Mono Progenitor Cell
Promyelocyte
Myelocytes
Metamyelocytes
Eosinophils

Fig. 1.8: Current understanding of eosinophilopoiesis in bone marrow

Granulogenesis

The morphologic characteristics of eosinophilopoiesis and the ontogeny of the formation of crystalloid granule have been partially investigated by analysis of eosinophil progenitors with use of electron microscopy and *in vitro* culture systems (166). This included the description of early commitment to the eosinophil phenotype by the appearance of numerous large immature granules in the cytoplasm (166). It has been reported that maturation of the crystalloid granules, in immature human eosinophilic myelocytes (*in vitro* culture system), may be accompanied by the formation of structures containing large irregular blocks of dense core materials surrounded by irregular, rope-like materials. Condensation of theses structures leads to the formation of central cores that are often irregularly shaped (167). The observation that immature granules appear during early eosinophil development was confirmed in later studies showing pro-MBP messenger RNA and protein expression in developing eosinophils *in vitro*, which localized to large immature granules containing "hollow" electron-lucent cores (168). None of these studies, however, examined the expression and pattern of granule storage of cytokines, in parallel with cationic proteins, in differentiating eosinophils.

Regulation of Eosinophilopoiesis

Activation of specific hemopoietic pathways in the bone marrow may contribute to the allergic response through increased production and traffic of lineage- committed inflammatory progenitor cells. In turn, lineage- committed progenitor may contribute to the disease process through increased production of key effector inflammatory cells, such as eosinophils. Eosinophilopoietic mechanisms involved in allergic inflammatory responses, beginning in the bone marrow, can be measured in the peripheral circulation and even in tissue compartments. This mechanism can be targeted by anti-allergy therapies, indeed, corticosteroid treatment in patients during an asthma exacerbation, leads to marked reduction in circulating and bone marrow Eo/B-CFU. Many of the effects of inhaled corticosteroids may be due to down-regulation of cytokine generation at the site of allergen challenge, which in turn inhibits signaling to distal sites such as the bone marrow. A direct effect of these drugs, however, on progenitors cannot be excluded since there is evidence (both *in vitro* and *in vivo*) that low, systemic levels of corticosteroid (10^{-9} M) can block the differentiation of the Eo/B progenitor in humans (169), and of the myeloid progenitor in dogs (170).

IV. Eosinophil-Derived Mediators

Introduction

It is now known that eosinophils synthesize, store and release a wide spectrum of proinflammatory mediators. The effector and regulatory functions of eosinophils are thought to be widely associated with eosinophil-derived proinflammatory mediators. Eosinophil-derived mediators include preformed basic proteins, *de novo* synthesized phospholipids, cytokines, various enzymes including proteases, and products of oxidative metabolism.

Eosinophil Granule Cationic Proteins

Eosinophil crystalloid granules contain four basic proteins known as major basic protein (MBP), eosinophil derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxides (EPO). As the name implies, MBP accounts for the majority (50%, approximately 250 pg/cell) of the granule protein found in guinea pig eosinophils and was named for that fact (171). Detailed molecular studies suggest that MBP is translated as a more neutral prepro-protein, to protect the cell from the toxic actions of the mature form, before it is taken up into the secondary granules and processed. Indeed, evidence now exists that pro-MBP is converted into mature MBP within granules during the process of eosinophil differentiation.

Recently a novel homolog of MBP has been also identified in human eosinophil granules. Although, it has similar biological activity to MBP, it is considerably less abundant and does not interact with MBP in a synergistic manner with respect to its cytotoxicity. Its role in eosinophil-driven histopathology is not yet known (172).

MBP is a 13.8-kDa arginine- and cysteine-rich polypeptide composed of 117 amino acids that feature alternating hydrophobic and hydrophilic sequences (173). MBP has the potential to act in a paracrine manner to modify the activity of other eosinophils. At low, non-cytotoxic concentrations, MBP is as effective as secretory IgA in evoking the exocytosis of EDN (174) and is also known to be a potent degranulator of basophils. Moreover, MBP is a helminthotoxin and cytotoxin and has been shown to possess bactericidal activity. The ability of MBP to damage target cells is due to its ability to increase membrane permeability through surface charge interactions rather than by the formation of distinct pores (175).

Unlike MBP, other cationic proteins (ECP, EDN and EPO) are predominantly stored in the granule matrix surrounding the core (176,177).

ECP, a zinc-rich single-chain peptide, has a molecular mass ranging between 16 and 21.4 kDa. (178). It shows a weak RNase activity, and exhibits a number of other properties. In addition to being bactericidal and helminthotoxic, ECP promotes the degranulation of mast cells (179,180). Even though, the action mechanism of ECP has not been studied in detail, it is believed that ECP exerts many of its effects by creating functional pores or channels that cross the plasma membrane of the target cells, which are neither voltage nor ion-sensitive (181). Interestingly, it has been shown that the cytotoxicity of ECP is not apparently dependent on its RNase activity (182).

EDN is an 18.5-kDa, single-chain polypeptide (179,183) with a pI of (8.9), which is approximately 10 to 100 times more acidic than either human MBP or ECP, due to a relatively lower number of arginine residues in the protein, which probably accounts for its reduced cytotoxicity (184). EDN is known to have relatively poor helminthotoxin and cytotoxin effects, and similar to ECP, possesss neurotoxic activity (185). The toxic effects of EDN have been linked to its marked and potent ribonuclease activity; nevertheless, it is not sufficient to account totally for its neurotoxicity (186). The high level of RNase activity associated with EDN suggests an additional but, as yet, undefined physiological function for the eosinophils.

EPO is a haem-containing protein composed of two subunits; a heavy chain of some 50- to 57-kDa and an 11- to 15-kDa light chain (187). The general consensus is that EPO is produced as a 79.5-kDa prepro-protein which is cleaved twice: first, by removal of the 13.8 kDa "pro" sequence to form an intermediate, and again resulting in two highly basic fragments corresponding to a light (12.7 kDa, pI = 10.8) and a heavy (53 kDa, pI = 10.7) chain (188). These chains may reassemble to form native EPO composed of a two-chain monomer or, possibly, a four-chain dimmer. Once released, EPO can elicit a number of effects, some of which are protective while others are potentially destructive. In particular, EPO inactivates peptido- leukotrienes and converts LTC_4 to all-*trans* isomers of LTB_4. LTB_4 is similarly inactivated by EPO but at a much slower rate (189). EPO also is bactericidal and in the presence of hydrogen peroxide and bromide, can catalyze the formation of hypobromous acid and the highly reactive singlet oxygen (190).

Lipid Mediators

Eosinophils have the capacity to synthesize an array of phospholipid-derived mediators that have widespread biological and pharmacological actions. Some of the more important products include PAF, LTC_4, prostaglandin-E_2 (PGE_2), thromboxane-B_2 (TXB_2), and lipoxin-A_4 (LXA_4). Lipid mediators play an important role in the pathogenesis of inflammatory and allergic response. Indeed, inhibiting the function of leukotrienes and 5-lipoxygenase (5-LO) and 5-LO-activating protein (FLAP), which are key proteins necessary for leukotriene synthesis, have been considered in therapeutic strategies (191,192).

Lipid mediators are generally divided into two major classes: metabolites of arachidonic acid, and 2-acetylated phospholipids, the best characterized of which is PAF (193). In most mammalian cells, the arachidonic acid and 2-acetylated phospholipids are synthesized from common precursor, 2-arachidonoyl-glycerophospholids that are normal constituents of the cell membrane (194,195) (fig. 5).

Human eosinophils are not only an important source of lipid mediators but also a major target of lipid mediators. In addition to their effect on various eosinophil functions, lipid mediators often influence their own production and release.

Eosinophil-derived cytokines, chemokines and growth factors

It is now clear that eosinophils synthesize up to 25 cytokines, chemokines, and growth factors, many of which are stored in their crystalloid granules (196,197). The recognition of cytokines/chemokines syntheses by eosinophils has led to a significant re-evaluation of their potential regulatory role in allergic inflammation. This is specially evident with regard to prolongation of allergic responses, activation of local inflammatory cells, and induction of further inflammatory cell infiltration from the blood into mucosal tissues such as the airways. The wide array of cytokines produced by eosinophils suggests that they may have the capacity to either exacerbate or modulate the inflammatory response. The majority of cytokines produced by eosinophils are likely to augment or maintain the Th2-like allergic reaction, particularly IL-1α (198), IL-2 (199), IL-3 (200), IL-4 (201), IL-5 (202), IL-6 (203), IL-9 (204), IL-10 (205), IL-16 (206), and GM-CSF (207).

In addition to the synthesis and release of regulatory cytokines, eosinophils also synthesize and release a number of chemokines, including eotaxin (208), MIP-1α (209), and RANTES (210), which may regulate the infiltration and recruitment of eosinophils and lymphocytes to the site of allergic inflammation. Eotaxin, an eosinophil-specific chemokine, is involved in chemoattraction of eosinophils and has been shown to be a highly specific and potent agonist for the induction of an eosinophilic influx during allergic response (211). Other chemokines produced by eosinophils including RANTES and MIP-1α, may be important regulators of local inflammatory response by exerting chemoattractant effects on leukocytes (212,213). RANTES is also known to induce eosinophil activation, degranulation and superoxide generation along with its effects on eosinophils chemotactic activity (214-216).

Eosinophil-derived growth factors are likely to contribute to the pathogenesis of allergic inflammation. Eosinophil-derived growth factors include, interferon-γ (IFNγ)

(217), GM-CSF (218), transforming growth factor α/β (TGFα/β) (219), tumor necrosis factor (TNF) (220), heparin-binding epidermal growth factor (HB-EGF) (221), nerve growth factor (NGF) (222), and SCF (223) (fig. 5).

V. Eosinophil Exocytosis and Mediator Release

Upon activation and recruitment to the site of inflammation, eosinophils have been shown to release their granule content to extracellular space. Various molecular mechanisms have been shown to underlie the eosinophil mediator release.

Cytolysis, Compound exocytosis, and Piecemeal Degranulation

Three mediator release patterns have been described in eosinophils; cytolysis or necrotic release, compound exocytosis, and piecemeal degranulation (PMD). Cytolysis is highlighted by degeneration of eosinophils and the loss of membrane integrity, which further leads to the release of intact granules to the extracellular space. There is evidence to suggest that eosinophil lysis and distribution of free eosinophil granules (as opposed to 'classical degranulation') is an important mechanism by which eosinophils affect their surroundings (224). Disrupted necrotic eosinophils have been observed ultrastructurally in the IgE-mediated late-phase reaction, (225) and also in skin lesions in the hypereosinophilic syndrome (226) and bullous pemphigoid (subepidermal blistering autoimmune disease of the elderly) (227) (fig. 9).

In compound exocytosis, a number of granules fuse intracellularly to form a large degranulation chamber or cavity, which in turn fuses with the cell membrane before discharging the contents to extracellular space (228) (fig. 9).

In physiological conditions, a more commonly observed mode of exocytotic mediator release in eosinophils, is PMD whereby stored mediators are selectively released from an intragranular pool leaving portions or all of the granules empty in the intact cell (229,230). PMD is characterized by the presence of a large number of small secretory vesicles in the cytoplasmic compartments, along with translucent crystalloid granules highlighted by their partial loss of content. In PMD, small secretory vesicles are thought to either bud from larger crystalloid granules to fuse with the plasma membrane, or are found within a separately mobilizable pool in the cytosol. The stimulation of these vesicles may result in selective release of granule mediators, including stored cytokines. *Ex vivo* studies have provided compelling evidence that the majority (67%) of tissue eosinophils undergo a PMD-type release in inflamed airways (231). Physiological evidence that eosinophils undergo selective PMD has been provided in studies showing that two eosinophil granule proteins, ECP and EDN, are differentially released during stimulation by complexes of different immunoglobulin subclasses (232,233) (fig. 9).

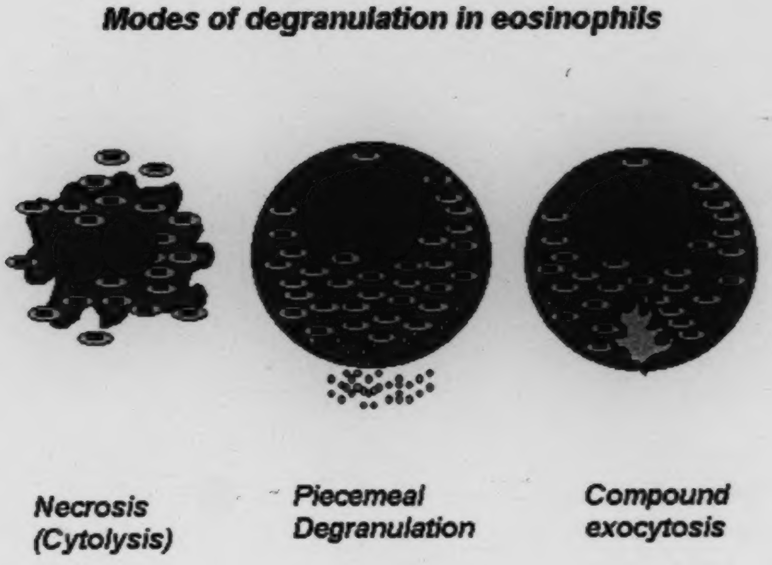

Modes of degranulation in eosinophils
Necrosis
(Cytolysis)
Piecemeal
Degranulation
Compound
exocytosis

Fig. 1.9: Three proposed modes of degranulation in human eosinophils. (Modified with permission from a schematic design by Dr. Redwan Moqbel)

SNARE fusion complex in eosinophil exocytosis

In yeast and neuronal cells, a highly homologous set of membrane-associated complex of proteins, known as SNAREs (SNAP Receptors), appears to regulate vesicular fusion. SNAREs were originally identified by their ability to bind the cytosolic fusion complex proteins SNAP (soluble NSF attachment protein) and NSF (N-ethylmaleimide sensitive factor)(234). SNARE proteins are subdivided into two functional groups: vesicular SNAREs (v-SNAREs) found on vesicular membranes, and target SNAREs (t-SNAREs), located on the target membrane. In neuronal cells, vesicle associated membrane protein-1 (VAMP-1) is one of the v-SNAREs, while syntaxin-1 and synaptosome-associated protein of 25 kDa (SNAP-25), located on the plasma membrane are postulated t-SNAREs. These potentially critical molecules for granule docking and fusion pore formation mediate granule-plasma membrane fusion leading to the extrusion of granule contents to the exterior of the cell (234). A functional SNARE complex is essential for neuronal cell exocytosis. The clostridial neurotoxins, tetanus (TeNT) and botulinum toxin serotypes (BoNT/A, B, C, D, E, F, and G), all of which contain zinc endopeptidase activity, inhibit exocytosis, by cleaving individual SNARE proteins specifically, thus, preventing their assembly as SNARE complexes (235,236). The pathological effects of TeNT and BoNT holotoxins are confined to neuronal cells since their heavy chain components require a ganglioside-binding site on the cell membrane, which is lacking in non-neuronal cells. These effects are most likely to be responsible for the spastic and flaccid paralysis induced by TeNT and BoNT toxin poisoning, respectively. SNARE isoforms have also been identified and shown to be a critical component of vesicular trafficking not only in exocytosis, but also in ER to Golgi (237,238), intra-Golgi (239), and endocytic transport (238).

More recently, new isoforms of SNAREs (VAMP-2, syntaxin-4 and SNAP-23) have been described in cells outside the nervous system, including mast cells, neutrophils and platelets (240). SNARE proteins have been implicated in translocation of GLUT-4 containing vesicles to the plasma membrane in insulin responsive tissues such as fat and muscle (241,242) and a role for SNARE proteins in exocytosis has also been identified in hemopoietic cells. Degranulation of mast cells (243,244) and platelets (245-247) appears to be dependent on SNAREs. In addition, treatment of macrophages with tetanus toxin prevents lysozyme secretion (248). Thus, SNARE molecules may be central to the process of exocytosis in eosinophils. Understanding the physiological role and activity of SNAREs in eosinophil mediator release may contribute to the development of new therapeutic strategies and at the current time members of our group are investigating the expression and function of members of SNAREs family in human eosinophils (fig. 10).

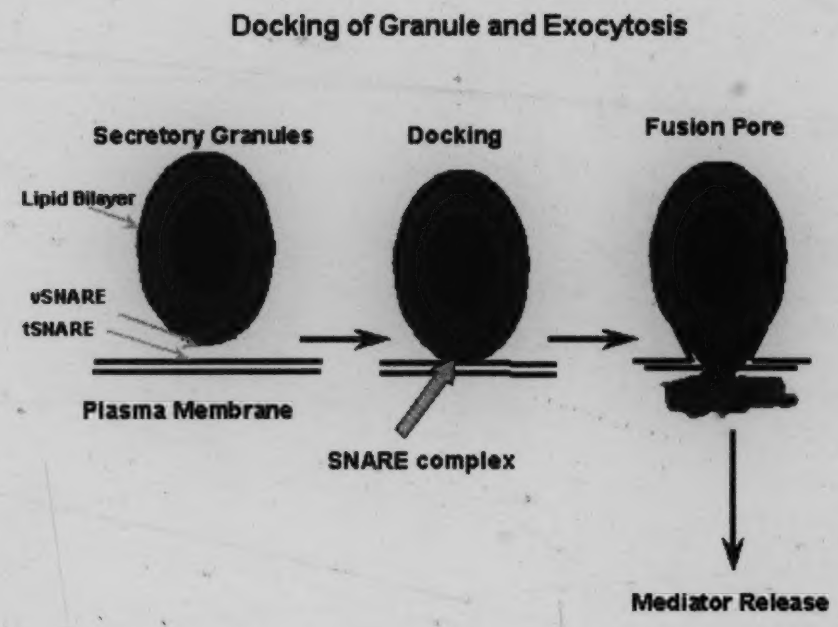

Docking of Granule and Exocytosis
Secretory Granules
Docking
Fusion Pore
Lipid Bilayer
vSNARE
tSNARE
Plasma Membrane
SNARE complex
Mediator Release

Fig. 1.10: Proposed model for SNARE molecules in exocytosis and granule docking.

Tetraspanin, CD63, in Eosinophil Exocytosis

The cell membrane is rather like an ocean afloat with many cell surface proteins, some in motion, while others are anchored to the cytoskeleton. Each individual protein may be capable of executing certain tasks (signaling, adhesion, exocytosis, etc.). It has been of extreme interest to scientists, however, to reveal the mechanisms and molecules involved in organization of these dissimilar cell surface proteins into functional mobile complexes, capable of united functions that they could not carry out as single molecules. There is a growing body of evidence suggesting that the members of an emerging family of proteins, the tetraspanins or transmembrane-4 superfamily (TM4SF), may serve just such an organizing function. Their ability to interact with many other signaling molecules and participate in activation, adhesion, and mediator release could all relate to the tetraspanin role as "molecular facilitators" that group together large molecular complexes and allow them, through stabilization, to function more efficiently (249,250).

Basic structural motifs of tetraspanins include the presence of four hydrophobic, putative transmembrane domains (TM1-TM4), forming a small and a large extracellular loop (EC1 and EC2), with short intracellular amino and carboxyl tails. These highly polar transmembrane-region residues may interact intramolecularly and play a part in the folding of the molecule; alternatively they may form intermolecular bonds and be involved in the assembly of complexes with other transmembrane molecules. In contrast to the hydrophobic domains, the extracellular domains of the family show a great deal of divergence in terms of length, sequence and degree of glycosylation.

Some tetraspanins have a nearly ubiquitous tissue distribution (CD9, CD63, CD81, CD82) whereas others are highly restricted, for example, to lymphoid and myeloid cells (CD53) of mature B cells (CD37) (249,250). Most TM4SF proteins can be found on the plasma membrane, and several are located in cell lameliopodia and filopdia, consistent with their role in motility (251,252). Many TM4SF proteins including CD9, CD37, CD53, CD63, CD81, CD82 and CD151, are also found in intracellular granules and vesicles (253-258). Although, the data currently available largely suggest a role for this superfamily in the regulation of cell development, proliferation, activation, adhesion and mediator release, the precise biochemical function of the TM4SF is not known (259-262).

CD63, previously known as lysosome-associated membrane protein-3 (lamp-3) and granulophysin (263,264), is a widely expressed glycoprotein member of the tetraspanin superfamily present on the surface of platelets, basophils, monocytes, macrophages and neutrophils (265-268) (fig. 11). The CD63 protein, utilized as a marker for lysosomal-type vesicles, contains a 'Gly-Tyr' lysosomal-targeting motif not found in other TM4SF proteins (269). CD63 is also known to be expressed in human eosinophils (270,271).

Since antibodies to CD63 inhibit the binding of monocytes to serum-coated plastic and also prevent the adhesion of neutrophils to endothelial cells (272), it has been proposed that CD63 may couple to intracellular signal transduction pathways and have a role in cellular adhesion. In endothelial cells, CD63 was identified as a component of Weibel-Palade bodies (273). These secretory granules undergo exocytosis upon appropriate stimulation. Interestingly, the adhesion protein, P-selectin and the von Willebrand factor also colocalize with CD63 to the Weibel-Palade bodies (273). Other studies have demonstrated that antibodies to CD63 induce neutrophil adhesion, which is probably mediated by LFA-1 (leukocyte function antigen) since antibodies to LFA-1 and

Ca^{2+} depletion block CD63 mediated adhesion (274). CD63 has also been shown to associate with tyrosine kinase activity in neutrophils (*Src*, *Lyn* and *Hck*) (269,275).

A number of other studies associated CD63 with early stages of melanoma. Interestingly, CD63 is strongly expressed on the cell surface in the primary stages of melanoma, but is weaker, or absent in the more malignant stages and in normal melanocytes (276,277). This has led to speculation that CD63 may limit the progression of melanoma. Studies on CD63-transfected 3T3 fibroblasts have shown that CD63 suppresses their growth in nude mice (278). These results have been further confirmed by studies showing that transfection of CD63 into human melanoma cells suppressed their growth and metastasis in nude mice whilst having no effect on the growth of melanoma cells *in vitro* (279). An association between CD63 with CD9 and CD81 in melanoma has been also demonstrated, which supports the idea that CD63, CD9 and CD81 may form a complex in the surface membrane of melanoma cells (280). The formation of these complexes is consistent with the view that the TM4SF molecules in general are involved in signal transduction. CD63 together with other members of TM4SF including CD9, CD81 and CD151, have been shown to specifically form a complex with phosphatidylinositol (PI) 4-Kinase. CD63–PI 4-kinase complexes were shown to almost entirely localize to intracellular space, and thus are considered distinct from other TM4SF–PI 4-kinase complexes (e.g. involving CD9), which are largely located in the plasma membrane. This may suggest that a specific subset of TM4SF proteins may recruit PI 4-kinase to specific membrane locations, and thereby influence phosphoinositide-dependent signaling (281).

There are further studies that associate CD63 with mediator release. In basophilic cells, the specific anti-CD63 antibody AD1 was shown to inhibit IgE-mediated histamine release (282). By immunoprecipitation, CD63 was shown to have close physical association with the FcεR1 in basophilic cells and a number of anti-human CD63 antibodies induced granular secretion from transfected rat basophilic leukemia (RBL) cells (283). CD63 may be sequestered in intracellular granules, prior to their translocation to the membrane during cell activation (284). In resting neutrophils, CD63 localizes to the membrane of azurophilic granules, while upon stimulation it translocates to the surface membrane (268). A similar phenomenon has been also shown in platelets and while, in resting platelets CD63 is present in dense granules and lysosomal membranes, following platelet activation, it translocates to the plasma membrane (285). Clearly there are a number of studies on CD63 function in various leukocyte populations, but its functions in human eosinophils yet to be known.

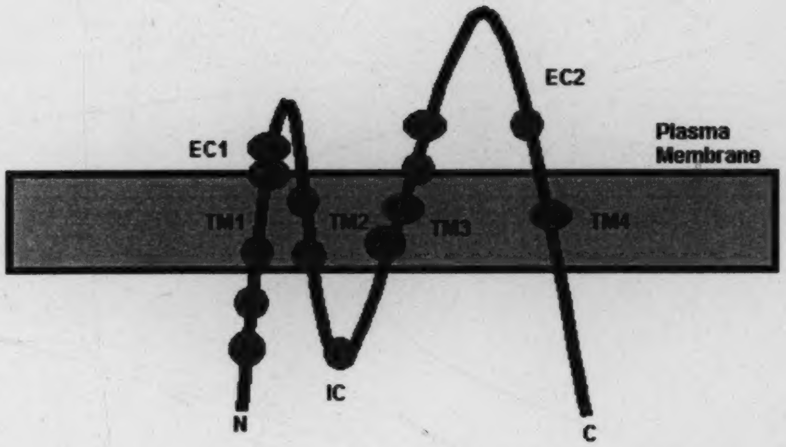

EC2
Plasma Membrane
EC1
TM1
TM2
TM3
TM4
IC
N
C

Fig. 1.11: Molecular structure of tetraspanin CD63, EC1 (extracellular loop 1), EC2 (extracellular loop 2), TM1-TM4 (transmembrane domains), IC (intracellular domain), N (N terminus), C (C terminus).

VI. Summary

Eosinophils are major effector cells in allergic inflammatory diseases particularly asthma. They have been shown to synthesize, store and release a wide range of proinflammatory mediators some of which are known to damage airway tissues. A large proportion of eosinophil-derived mediators are stored in unique crystalloid granules. Upon recruitment to the site of inflammation and activation, eosinophil-derived mediators are released to the extracellular space. Mechanisms underlying mediator expression, storage, mobilization and release in eosinophils remain largely unknown.

VII. Hypotheses

We hypothesize that:

1. ***In the early stages of eosinophil maturation, eosinophil-derived mediators are expressed and stored within structures that further evolve to crystalloid granules.***
2. ***Mediators stored within crystalloid granules could be selectively and rapidly mobilized and released to extracellular space upon agonist stimulation.***
3. ***CD63 is expressed in eosinophils and is associated with selective mediator mobilization and release in agonist-activated eosinophils of asthmatic individuals.***

VIII. Objective

1. *To identify and optimize necessary techniques in studying mediator expression, and localization in human eosinophils.*

2. *To determine cytokine and granule protein expression and storage during eosinophil maturation from cord blood-derived CD34$^+$ progenitors.*
3. *To determine mechanisms underlying selective mobilization and release of intracellularly stored mediators in response to agonist activation in human peripheral blood eosinophils.*
4. *To determine the CD63 expression in resting and agonist-activated human peripheral blood eosinophils and its association with selective mediator mobilization and release*

IX. References

1. Ciba Guest Symposium. 1959. Terminology, definitions, and classifications of chronic pulmonary emphysema and related conditions. *Thorax* 14:286-299.
2. American Thoracic Society. 1962. Definitions and classifications of chronic bronchitis, asthma, and pulmonary emphysema. *AARD* 85:762-768.
3. Barbee, R.A., and S. Murphy. 1998. The natural history of asthma. *J. Allergy Clin. Immunol.* 102:S65-72.
4. Holgate, S.T., C. Robinson, M. K. Church, and P.H. Howarth. 1985. The release and role of inflammatory mediators in asthma. *Clin. Immunol. Rev.* 4:241-288.
5. National Asthma Education and Prevention Program. Expert Panel Report: guidelines for the diagnosis and management of asthma. Bethesda (MD): NIH/National Heart, Lung, and Blood Institute, 1991. Publication No. 91-3042.
6. National Asthma Education and Prevention Program. Expert Panel Report 2: guidelines for the diagnosis and management of asthma: clinical practice guidelines. Bethesda (MD): NIH/National Heart, Lung, and Blood Institute, 1997. Publication No. 97-4051.
7. Holgate, S.T. 1997. Asthma: a dynamic disease of inflammation and repair. *In* The rising trends in asthma. D. Chadwick, G. Cardew, editors. Ciba Foundation Symposium 206. John Wiley and Sons, Chichester, UK. 5-34.
8. Snapper, J.R., and K.L. Brigham. 1984. Inflammation and airway reactivity. *Exp. Lung Res.* 6:83-89.
9. Cutz, E., H. Levison, and D.M. Cooper. 1978. Ultrastructure of airways in children with asthma. *Histopathology* 2:407-421.

10. Barnes, P.J. 1992. Frontiers in medicine: new aspects of asthma. *J. Intern. Med.* 231:453-461.

11. Romagnani, S. 1991. Human Th1 and Th2: doubt no more. *Immunol. Today.* 12:256-257.

12. Stampfli, M.R., and M. Jordana. 1998. Eosinophilia in antigen-induced airways inflammation. *Can. Respir. J.* A:31A-5A.

13. Denburg, J.A. 1998. Basophils and mast cells in airway inflammation and asthma. *Can. Respir. J.* 5 A:41A-4A.

14. Holgate, S.T., D.E. Davies, P.M. Lackie, S.J. Wilson, S.M. Puddicombe, and J.L. Lordan. 2000. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J. Allergy. Clin. Immunol.* 105:193-204.

15. Tremblay, G.M., J. Chakir, L.P. Boulet, M. Jordana, C.D. Richards, and J. Gauldie. 1998. Bronchial myofibroblasts and tissue remodelling in asthma. *Can. Respir. J.* 5:59-61.

16. Chung, K.F. 2000. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *Eur. Respir. J.* 15:961-968.

17. Jung, K.S., and H.S. Park. 1999. Evidence for neutrophil activation in occupational asthma. *Respirology* 4:303-306.

18. Kon, O.M., and A.B. Kay. 1999. T cells and chronic asthma. *Int. Arch. Allergy. Immunol.* 118:133-135.

19. Le Gros, G., K. Erb, N. Harris, J. Holloway, K. McCoy, and F. Ronchese. 1998. Immunoregulatory networks in asthma. *Clin. Exp. Allergy* 28:117-118.

20. Caldwell, C.W., and R.A. Burningham. 1975. Lymphocyte physiology and function. *Mil. Med.* 140:846-852.

21. Meuwissen, H.J., O. Stutman, and R.A Good. 1969. Functions of the lymphocytes. *Semin. Hematol.* 6(1):28-66.

22. Yssel, H., and H. Groux. 2000. Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. *Int. Arch. Allergy Immunol.* 121(1):10-18.

23. Umetsu, D.T., and R.H. DeKruyff. 1997. TH1 and TH2 CD4+ cells in human allergic diseases. *J. Allergy Clin. Immunol.* 100(1):1-6.

24. HayGlass, K.T., M. Wang, R.S. Gieni, C. Ellison, and J. Gartner. 1996. *In vivo* direction of CD4 T cells to Th1 and Th2-like patterns of cytokine synthesis. *Adv. Exp. Med. Biol.* 409:309-316.

25. Abbas, A.K., K.M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787-793.

26. Romagnani, S. 2000. T-cell subsets (Th1 versus Th2). *Ann. Allergy Asthma Immunol.* 85:9-18.

27. De Carli, M., M.M. D'Elios, G. Zancuoghi, S. Romagnani , and G. Del Prete. 1994. Human Th1 and Th2 cells: functional properties, regulation of development and role in autoimmunity. *Autoimmunity.* 18:301-308.

28. Morel, P.A., and T.B. Oriss. 1998. Crossregulation between Th1 and Th2 cells. *Crit. Rev. Immunol.* 18:275-303.

29. Secrist, H., C.J. Chelen, Y. Wen, J.D. Marshall, and D.T. Umetsu. 1993. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J. Exp. Med.* 178:2123-2130.

30. Medina, E., and C.A. Guzman. 2000. Modulation of immune responses following antigen administration by mucosal route. *FEMS Immunol. Med. Microbiol.* 27:305-311.

31. Romagnani, S. 1995. Biology of human TH1 and TH2 cells. *J. Clin. Immunol.* 15:121-129.

32. Singh, V.K., S. Mehrotra, and S.S. Agarwal. 1999. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol. Res.* 20:147-161.

33. Sornasse, T., P.V. Larenas, K.A. Davis, J.E. de Vries, and H. Yssel. 1996. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4+ T cells, analyzed at the single-cell level. *J. Exp. Med.* 184:473-483.

34. Maggi, E. 1998. The TH1/TH2 paradigm in allergy. *Immunotechnology.* 3:233-244.

35. Bellanti, J.A. 1998. Cytokines and allergic diseases: clinical aspects. *Allergy Asthma Proc.* 19:337-341.

36. MacDonald, T.T. 1999. Effector and regulatory lymphoid cells and cytokines in mucosal sites. *Curr. Top. Microbiol. Immunol.* 236:113-135.

37. Warner, J.O. 1998. Bronchial hyperresponsiveness, atopy, airway inflammation, and asthma. *Pediatr. Allergy Immunol.* 9:56-60.

38. Knox, A.J., L. Pang, Y.M. Zhu. 2000. Targeting remodeling in respiratory inflammation. *Curr. Opin. Investig. Drugs.* 1:428-434.

39. Busse, W.W. 1998. Inflammation in asthma: the cornerstone of the disease and target of therapy. *Allergy Clin. Immunol.* 102:S17-22.

40. Robinson, D.S., R. Damia, K. Zeibecoglou, S. Molet, J. North, T. Yamada, A. Barry Kay, and Q. Hamid. 1999. CD34(+)/interleukin-5 R-alpha messenger RNA+ cells in the bronchial mucosa in asthma: potential airway eosinophil progenitors. *Am. J. Respir. Cell. Mol. Biol.* 20:9-13.

41. Linden, M., C. Svensson, M. Andersson, L. Greiff, E. Andersson, J.A. Denburg, and C.G. Persson. 1999. Circulating eosinophil/basophil progenitors and nasal mucosal cytokines in seasonal allergic rhinitis. *Allergy.* 54:212-219.

42. Engelhardt, J.F., H. Schlossberg, J.R. Yankaskas, and L. Dudus. 1995. Progenitor cells of the adult human airway involved in submucosal gland development. *Development.* 121:2031-2046.

43. Jager L. 1997. The role of bronchial epithelium in asthma. *J. Investig. Allergol. Clin. Immunol.* 7:277-278.

44. Velden, V.H., and H.F. Versnel. 1998. Bronchial epithelium: morphology, function and pathophysiology in asthma. *Eur. Cytokine Netw.* 9:585-597.

45. Roche, W.R. 1991. Fibroblasts and asthma. *Clin. Exp. Allergy.* 21:545-548.

46. Gizycki, M.J., E. Adelroth, A.V. Rogers, P.M. O'Byrne, and P.K. Jeffery. 1997. Myofibroblast involvement in the allergen-induced late response in mild atopic asthma. *Am. J. Respir. Cell. Mol. Biol.* 16:664-673.

47. Schmidt, D., K.F. Rabe. 2000. Immune mechanisms of smooth muscle hyperreactivity in asthma. *J. Allergy. Clin. Immunol.* 105:673-682.

48. Hirst, S.J. 2000. Airway smooth muscle as a target in asthma. *Clin. Exp. Allergy*. 30:54-59.

49. Lemanske, R.F. Jr. 2000. Inflammatory events in asthma: an expanding equation. *J. Allergy Clin. Immunol.* 105:S633-636.

50. Fahy, J.V., D.B. Corry, and H.A. Boushey. 2000. Airway inflammation and remodeling in asthma. *Curr. Opin. Pulm. Med.* 6:15-20.

51. Schor, H., G.G. Vaday, and O. Lider. 2000. Modulation of leukocyte behavior by an inflamed extracellular matrix. *Dev. Immunol.* 7:227-238.

52. Vaday, G.G., and O. Lider. 2000. Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. *J. Leukoc. Biol.* 67:149-159.

53. Mautino, G., F. Capony, J. Bousquet, and A.M. Vignola. 1999. Balance in asthma between matrix metalloproteinases and their inhibitors. *J. Allergy Clin. Immunol.* 104:530-533.

54. Goldring, K., and J.A. Warner. 1997. Cell matrix interactions in asthma. *Clin. Exp. Allergy*. 27:22-27.

55. Ottonello, L., P. Dapino, M. Amelotti, P. Barbera, N. Arduino, M. Bertolotto, and F. Dallegri. 1998. Activation of neutrophil respiratory burst by cytokines and chemoattractants. Regulatory role of extracellular matrix glycoproteins. *Inflamm. Res.* 47:345-350.

56. Kita, H., S. Horie, and G.J. Gleich. 1996. Extracellular matrix proteins attenuate activation and degranulation of stimulated eosinophils. *J. Immunol.* 156:1174-1181.

57. Roberts, C.R., and A.K. Burke. 1998. Remodeling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. *Can. Respir. J.* 5:48-50.

58. Sanerkin, N.G. 1972. Reversibility of airways obstruction. *Lancet*. 2:185-6.

59. Fernandez Rego, G., and A. Roman Rojo. 1985. Asthma and irreversible airflow obstruction. *Thorax*. 40:80.

60. Hansen, E.F., K. Phanareth, L.C. Laursen, A. Kok-Jensen, and A. Dirksen. 1999. Reversible and irreversible airflow obstruction as predictor of overall mortality in asthma and chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care. Med.* 159:1267-1271.

61. Fish, J.E., and S.P. Peters. 1999. Airway remodeling and persistent airway obstruction in asthma. *J. Allergy Clin. Immunol.* 104:509-516.

62. Pare, P.D., C.R. Roberts, T.R. Bai, and B.J. Wiggs. 1997. The functional consequences of airway remodeling in asthma. *Monaldi Arch. Chest Dis.* 52:589-596.

63. Stewart, A.G., P.R. Tomlinson, and J.W. Wilson. 1995. Regulation of airway wall remodeling: prospects for the development of novel antiasthma drugs. *Adv. Pharmacol.* 33:209-253.

64. Hungerford, G.D., H.B. Williams, and B. Gandevia. 1977. Bronchial walls in the radiological diagnosis of asthma. *Br. J. Radiol*. 50:783-787.

65. Park, J.W., Y.K. Hong, C.W. Kim, D.K. Kim, K.O. Choe, and C.S. Hong. 1997. High-resolution computed tomography in patients with bronchial asthma: correlation with clinical features, pulmonary functions and bronchial hyperresponsiveness. *J. Investig. Allergol. Clin. Immunol.* 7:186-192.

66. Hegele, R.G. 2000. The pathology of asthma: brief review. *Immunopharmacology.* 48:257-262.

67. Laitinen, A., E.M. Karjalainen, A. Altraja, and L.A. Laitinen. 2000. Histopathologic features of early and progressive asthma. *J. Allergy Clin. Immunol.* 105:509-513.

68. Djukanovic, R. 2000. Asthma: A disease of inflammation and repair. J. *Allergy Clin. Immunol.* 105:522-526.

69. DuBuske, L.M. 1999. The link between allergy and asthma. *Allergy Asthma Proc.* 20:341-345.

70. Daser, A., H. Mitchison, A. Mitchison, and B. Muller. 1996. Non-classical-MHC genetics of immunological disease in man and mouse. The key role of pro-inflammatory cytokine genes. *Cytokine* 8:593-597.

71. Holt, P.G., C. Macaubas, P.A. Stumbles, and P.D. Sly. 1999. The role of allergy in the development of asthma. *Nature*. 402:12-17.

72. Rabe, K.F. 1998. Mechanisms of immune sensitization of human bronchus. *Am. J. Respir. Crit. Care Med.* 158:161-170.

73. Holt, P.G. 1993. Regulation of antigen-presenting cell function(s) in lung and airway tissues. *Eur. Respir. J.* 6:120-129.

74. Machado, L., G. Stalenheim, and P. Malmberg. 1986. Early and late allergic bronchial reactions: physiological characteristics. *Clin. Allergy*. 16:111-117.

75. Picado, C. 1992. Early and late-phase asthmatic reactions: a hypothesis. *Allerg.* 47:331-333.

76. Bingham, C.O., and K.F. Austen. 2000. Mast-cell responses in the development of asthma. *J. Allergy Clin. Immunol*. 105:527-534.

77. Burke, L.A., J.R. Wilkinson, C.J. Howell, and T.H. Lee. 1991. Interactions of macrophages and monocytes with granulocytes in asthma. *Eur. Respir. J.* 13:85-90.

78. Lee, B.W., R.S. Geha, and D.Y. Leung. 1988. IgE response and its regulation in allergic diseases. *Pediatr. Clin. North Am.* 35:953-967.

79. Wasserman, S.I. 1994. Mast cells and airway inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 150:39-41.

80. Wasserman, S.I. 1987. The regulation of inflammatory mediator production by mast cell products. *Am. Rev. Respir. Dis.* 135:46-48.

81. Befus, D. 1987. The role of the mast cell in allergic bronchospasm. *Can. J. Physiol. Pharmacol.* 65:435-441.

82. Kaliner, M. 1987. Mast cell mediators and asthma. *Chest*. 91:171-176.

83. Konig, W., W. Schonfeld, J. Knoller, and M. Koller. 1988. The role of lipid mediators in asthma (current views and perspectives). *Allergol. Immunopathol.* 16:289-298.

84. Lewis, R.A. 1985. Leukotrienes and other lipid mediators of asthma. *Chest*. 87:5-10.

85. Hargreave, F.E. 1989. Late-phase asthmatic responses and airway inflammation. *J. Allergy Clin. Immunol.* 83:525-527.

86. Lemanske, R.F. Jr. 1991. The late phase response: clinical implications. *Adv. Intern. Med.* 36:171-193.

87. Hellewell, P.G. 1999. Adhesion molecule strategies. *Pulm. Pharmacol. Ther.* 12:137-41.

88. Schleimer, R.P., and B.S. Bochner. 1998. The role of adhesion molecules in allergic inflammation and their suitability as targets of antiallergic therapy. *Clin. Exp. Allergy*. 28:15-23.

89. Tonnel, A.B., P. Gosset, S. Molet, I. Tillie-Leblond, P. Jeannin, and M. Joseph. 1996. Interactions between endothelial cells and effector cells in allergic inflammation. *Ann. N.Y. Acad. Sci.* 796:9-20.

90. Lassalle, P., P. Gosset, Y. Delneste, A. Tsicopoulos, A. Capron, M. Joseph, and A.B. Tonnel. 1993. Modulation of adhesion molecule expression on endothelial cells during the late asthmatic reaction: role of macrophage-derived tumour necrosis factor-alpha. *Clin. Exp. Immunol.* 94:105-110.

91. Tonnel, A.B., P. Lassalle, and Y. Delneste. 1993. Role of endothelial cells in the pathogenesis of bronchial asthma. *Ann. Allergy*. 71:306-311.

92. Pilewski, J.M., and S.M. Albelda. 1995. Cell adhesion molecules in asthma: homing, activation, and airway remodeling. *Am. J. Respir. Cell. Mol. Biol.* 12:1-3.

93. Hirata, N., H. Kohrogi, H. Iwagoe, E. Goto, J. Hamamoto, K. Fujii, T. Yamaguchi, O. Kawano, and M. Ando. 1998. Allergen exposure induces the expression of endothelial adhesion molecules in passively sensitized human bronchus: time course and the role of cytokines. *Am. J. Respir. Cell Mol. Biol.* 18:12-20.

94. Boyce, J.A. 1997. The pathobiology of eosinophilic inflammation. *Allergy Asthma Proc.* 18:293-300.

95. Gleich, G.J. 2000. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* 105:651-663.

96. Jones, T.W. 1846. The blood-corpuscle considered in its different phases of development in the animal series. Memoir I Vertebrata. *Phil. Trans. R. Soc. Lond.* 136: 63-87.

97. Archer, R.K. 1963. The Eosinophil Leukocytes. Blackwell Scientific. Oxford.

98. Spry, C.J. 1988. Eosinophils. A Comprehensive Review, and Guide to the Scientific and Medical Literature. Oxford University Press, Oxford, 502pp.

99. Brown, T.R. 1898. Studies on trichinosis with especial reference to the increase of the eosinophilic cells in the blood and muscle, the origin of these cells and their diagnostic importance. *J. Exp. Med.* 3: 315-3

100. Ehrlich, P. 1879. Ueber die specifischen granulationen des blut. *Arch. Anat. Physiol. Lpz. 3 Physiol. Abstr.* 571-579.

101. Butterworth, A.E. and K.J.I. Thorne. 1993. Eosinophils and parasitic diseases. *In* Immunopharmacology of Eosinophils. H. Smith and R.M. Cook, editors. Academic Press, London. 119-150.

102. Allen, J.N. and W.B. Davis. 1994. Eosinophilic lung diseases. *Am. J. Respir. Crit. Care Med.* 150: 1423-1438.

103. Boguniewicz, M. 1997. Advances in the understanding and treatment of atopic dermatitis. *Curr. Opin. Pediatr.* 9:577-81.

104. Klementsson, H. 1992. Eosinophils and the pathophysiology of allergic rhinitis. *Clin. Exp. Allergy* 22:1058-64.

105. Kay, A.B., L. Barata, Q. Meng, S.R. Durham, and S. Ying. 1997. Eosinophils and eosinophil-associated cytokines in allergic inflammation. *Int. Arch. Allergy Immunol.* 113:196-9.

106. Ovington, K.S., and C.A. Behm. 1997. The enigmatic eosinophil: investigation of the biological role of eosinophils in parasitic helminth infection. *Mem. Inst. Oswaldo Cruz.* 2:93-104.

107. Patterson, R., and K.E. Harris. 1999. Idiopathic anaphylaxis. *Allergy Asthma Proc.* 20:311-5.

108. Walsh, G.M. 1999. Advances in the immunobiology of eosinophils and their role in disease. *Crit. Rev. Clin. Lab. Sci.* 36:453-96.

109. Sokol, R.J., G. Hudson, M.J. Brown, J. Wales, and N.T. James. 1988. Hypereosinophilic syndrome: A case report and morphometric study. *Acta. Haematol.* 79;107-111.

110. Dvorak, A.M. 1991. Subcellular morphology and biochemistry of eosinophils. *In* Blood Cell Biochemistry: Megakaryocytes, Platelets, Macrophages and Eosinophils. J.R. Harris, editor. Plenum Press, London. 237-344.

111. Dvorak, A.M., and P.F. Weller. 2000. Ultrastructural analysis of human eosinophils. *Chem. Immunol.* 76:1-28.

112. Zucker Franklin, D. 1980. Eosinophil structure and maturation. *In* The Eosinophil in Health and Disease. A.A.F. Mahmoud and K.F. Austen, editors. Grune and Stratton, New York. 43-60.

113. Saito, H., K. Hatake, A.M. Dvorak, K.M. Leiferman, A.D. Donnenberg, N. Arai, K. Ishizaka, and T. Ishizaka. 1988. Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc. Natl. Acad. Sci. USA*. 5:2288-2292.

114. Frigas, E., D.A. Loegering, G.J. Gleich. 1980. Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab. Invest.* 42:35-43.

115. Gleich, G.J., E. Frigas, D.A. Loegering, D.L. Wassom, and D. Steinmuller. 1979. Cytotoxic properties of the eosinophil major basic protein. *J. Immunol.* 123:2925-2927.

116. Ackerman, S.J., G.J. Gleich, D.A. Loegering, B.A. Richardson, and A.B. Butterworth. 1985. Comparative toxicity of purified human eosinophil granule cationic proteins for schistosomula of *S*chistosoma mansoni. *Am. J. Trop. Med. Hyg.* 34:735-745.

117. Butterworth, A.E., D.L. Wassom, G.J. Gleich, D.A. Loegering, and J.R. David. 1979. Damage to schistosomula of Schistosoma mansoni induced directly by eosinophil major basic protein. *J. Immunol.* 122:221-229. 26.

118. Kierszenbaum, E., S.J. Ackerman, and G.J. Gleich. 1981. Destruction of bloodstream forms of Trypanosoma cruzi by eosinophil granule major basic protein. *Am. J. Trop. Med. Hyg.* 30:775-779.

119. Peters, M.S., M. Rodriguez, and G.J. Gleich. 1986. Localization of human eosinophil granule major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin by immunoelectron microscopy. *Lab. Invest.* 54:656-662.

120. Lacy, P., and R. Moqbel. 2000. Eosinophil cytokines. *Chem. Immunol.* 76:134-55.

121. Newman, T.M., M. Tian and B.D. Gomperts. 1996. Ultrastructural characterization of tannic acid-arrested degranulation of permeabilized guinea pig eosinophils stimulated with GTPγS. *Eur. J. Cell Biol.* 70:209-220.

122. Dvorak, A.M., L. Letourneau, G.R. Login, P.F. Weller and S.J. Ackerman. 1988. Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to a distinct crystalloid-free granule population in mature human eosinophils. *Blood.* 72: 150-158.

123. Weller, P.F., E.J. Goetzl, and K.F. Austen. 1980. Identification of human eosinophil lysophospholipase as the constituent of Charcot-Leyden crystals. *Proc. Natl. Acad. Sci. USA.* 77: 7440-7443.

124. Ackerman, S.J., D.A. Loegering, and G.J. Gleich. 1980. The human eosinophil Charcot-Leyden crystal protein: biochemical characteristics and measurement by radioimmunoassay. *J. Immunol.* 125: 2118-2126.

125. Parmley, R.T., and S.S. Spicer. 1974. Cytochemical and ultrastructural identification of a small type granule in human late eosinophils. *Lab. Invest.* 30: 557-567.

126. Iozzo, R.V., A.H. MacDonald, and T.N. Wight. 1982. Immunoelectron microscopic localization of catalase in human eosinophilic leukocytes. *J. Histochem. Cytochem.* 30:697-701.

127. Weller, P.F., and A.M. Dvorak. 1985. Arachidonic acid incorporation by cytoplasmic lipid bodies of human eosinophils. *Blood.* 65:1269-1274.

128. Weller, P.F., R.A. Monahan Earley, H.R. Dvorak, and A.M. Dvorak. 1991. Cytoplasmic lipid bodies of human eosinophils. Subcellular isolation and analysis of arachidonate incorporation. *Am. J. Pathol.* 138:141-148.

129. Beasley, R., W.R. Roch, J.A. Roberts, and S.T. Holgate. 1989. Cellular events in the bronchi in mild asthma and after provocation. *Am. Rev. Respir. Dis.* 139:806-817.

130. Salvato, G. 1968. Some histological changes in chronic bronchitis and asthma. *Thorax.* 23:168-172.

131. Dunnill, M.S. 1960. The pathology of asthma, with special reference to changes in the bronchial mucosa. *J. Clin. Pathol.* 13:27-33.

132. Filley, W.V., K.E. Holley, G.M. Kephart, and G.J. Gleich. 1982. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet*. 2:11-16.

133. Wardlaw, A.J., S. Dunnette, G.J. Gleich, J.V. Collins, and A.B. Kay. 1988. Eosinophils and mast cells in bronchoalveolar lavage in mild asthma: relationship to bronchial hyperactivity. *Am. Rev. Respir. Dis.* 137:62-69.

134. Rossi, G.A., E. Crimi, S. Lantero, P. Gianiorio, S. Oddera, P. Crimi, and V. Brusasco. 1991. Late-phase asthmatic reaction to inhaled allergen is associated with early recruitment of eosinophils in the airways. *Am. Rev. Respir. Dis.* 144:379-383.

135. Aalbers, R., H.F. Kauffman, B. Vrugt, G.H. Koeter, and J.G. de Monchy. 1993. Allergen-induced recruitment of inflammatory cells in lavage 3 and 24 h after challenge in allergic asthmatic lungs. *Chest*. 103:1178-1184.

136. Mapp, C., P. Boschetto, D. Miotto, E. De Rosa, and L.M. Fabbri. 1999. Mechanisms of occupational asthma. *Ann. Allergy Asthma Immunol.* 83:645-664.

137. Coyle, A.J., D. Uchida, S.J. Ackerman, W. Mitzner, and C.G. Irvin. 1994. Role of cationic proteins in the airway. Hyperresponsiveness due to airway inflammation. *Am. J. Respir. Crit. Care Med.* 150:S63-71.

138. Gundel, R.H., L.G. Letts, and G.J. Gleich. 1991. Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J. Clin. Invest.* 87:1470-1473.

139. Lefort, J., M.A. Nahori, C. Ruffie, B.B. Vargaftig, and M. Pretolani. 1996. *In vivo* neutralization of eosinophil-derived major basic protein inhibits antigen-induced bronchial hyperreactivity in sensitized guinea pigs. *J. Clin. Invest.* 97:1117-1121.

140. Motojima, S., E. Frigas, D.A. Loegering, and G.J. Gleich. 1989. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium *in vitro*. *Am. Rev. Respir. Dis.* 139:801-805.

141. Leckie M.J., A. ten Brinke, J. Khan, Z. Diamant, B.J. O'Connor, C.M. Walls, A.K. Mathur, H.C. Cowley, K.F. Chung, R. Djukanovic, T.T. Hansel, S.T. Holgate, P.J. Sterk, and P.J. Barnes. 2000. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet.* 356:2144-2148.

142. Ogawa, M. 1994. Hematopoiesis. *J. Allergy Clin. Immunol.* 94:645-650.

143. Bedi, A., and S.J. Sharkis. 1995. Mechanisms of cell commitment in myeloid cell differentiation. *Curr. Opin. Hematol.* 2:12-21.

144. Till, J.E., E.A. McCullogh, and L. Siminovitch. 1964. A stochastic model of stem cell proliferation, based on growth of spleen-forming cells. *Proc. Natl. Acad. Sci. USA.* 51:29-36.

145. Humphries, R.K., A.C. Eaves, and C.J. Eaves. 1981. Self-renewal of hemopoietic stem cells during mixed colony formation *in vitro*. *Proc. Natl. Acad. Sci. USA.* 78:3629-3633.

146. Nakahata, T., A.J. Gross, and M. Ogawa. 1982. A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. *J. Cell Physiol.* 113:455-458.

147. Nakahata, T., and M. Ogawa. 1982. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential colonies. *Proc. Natl. Acad. Sci. USA.* 79:3843-3847.

148. Civin, C., L.C. Strauss, C. Brovall, M.J. Fackler, J.F. Schwartz, and J.H. Shaper. 1984. Antigenic analysis of hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-la cells. *J. Immunol.* 133:157-165.

149. Sutherland, D.R., and A. Keating. 1992. The CD34 antigen: structure, biology and potential clinical applications. *J. Hemother.* 1:115-129.

150. Majdic, 0., J. Stockl, W.F. Pickl, J. Bohuslav, H. Strobl, C. Scheinecker, H. Stockinger, W. Knapp. 1994. Signalling and induction of enhanced cytoadhesiveness via the hematopoietic progenitor cell surface molecule, CD34. *Blood.* 83:1226-1234.

151. Strobl, H., M. Takimoto, O. Majdic, G. Fritsch, C. Scheinecker, P. Hocker, and W. Knapp. 1993. Myeloperoxidase expression in CD34 + normal human hematopoietic cells. *Blood.* 82:2069-2078.

152. Metcalf, D. 1989. The molecular control of cell division, differentiation, commitment, and maturation of hematopoietic cells. *Nature.* 339:27-30.

153. Denburg, J.A., H. Messner, B. Lim, N. Jamal, S. Telizyn, and J. Bienenstock. 1985. Clonal origin of human basophil/mast cells from circulating multipotent hemopoietic progenitors. *Exp. Hematol.* 13:185-188.

154. Denburg, J.A., S. Telizyn, A. Belda, J. Dolovich, and J. Bienenstock. 1985. Increased numbers of circulating basophil progenitors in atopic patients. *J. Allergy. Clin. Immunol.* 76:466-472.

155. Denburg, J.A., S. Telizyn, A. Belda, H. Messner, B. Lim, N. Jamal, S.J. Ackerman, G. J. Gleich, and J. Bienenstock. 1985. Heterogeneity of human peripheral blood eosinophil-type colonies: Evidence of a common basophil-eosinophil progenitor. *Blood.* 66:312-318.

156. Ackermann, S.J., G.M. Kephart, T.M. Habermann, P.R. Greipp, and G.J. Gleich. 1983. Localization of eosinophil granule major basic protein in human basophils. *J. Exp. Med.* 158:946-961.

157. Ackermann, S.J., G.J. Weil, and G.J. Gleich. 1982. Formation of Charcot-Leyden crystals by human basophils. *J. Exp. Med.* 155:1597-1609.

158. Boyce, J.A., D. Friend, R. Matsumoto, K.F. Austen, and W.F. Owen. 1995. Differentiation *in vitro* of hybrid eosinophil/basophil granulocytes: Autocrine function of an eosinophil developmental intermediate. *J. Exp. Med.* 182:49-57. D

159. Denburg, J.A. 1990. Cytokine-induced human basophil/mast cell growth and differentiation *in vitro*. *Springer Semin. Immunopathol.* 12:401-414.

160. Denburg JA. 1992. Basophil and mast cell lineages *in vitro* and *in vivo*. *Blood.* 79:846-860.

161. Clutterbuck. E.J., and C.J. Sanderson. 1988. Human eosinophil hematopoiesis studied *in vitro* by means of murine eosinophil differentiation factor (IL-5): Production of functionally active eosinophils from normal human bone marrow. *Blood.* 71:646-651.

162. Sanderson, C.J., D.J. Warren, and M. Strath. 1985. Identification of a lymphokine that stimulates eosinophilic differentiation *in vitro*: Its relationship to interleukin 3 and functional properties of eosinophils produced in cultures. *J. Exp. Med.* 162:60-74.

163. Valent, P., L.K. Ashman, W. Hinterberger, F. Eckersberger, O. Majdic, K. Lechner, P. Bettleheim. 1989. Mast cell typing. Demonstration of a distinct hematopoietic cell type and evidence of immunophenotypic relationship to mononuclear phagocytes. *Blood.* 73:1778-1785.

164. Ema, H., T. Suda, K. Nagayoshi, Y. Miura, C.I. Civin, and H. Nakauchi. 1990. Target cells for granulocyte macrophage colony-stimulating factor, interleukin-3 and interleukin-5 in the differentiation of eosinophils and neutrophils. *Blood.* 76:1956-1961.

165. Kobayashi, H. 1993. Effect of c-kit ligand (stem cell factor) in combination with interleukin-5, granulocyte- macrophage colony-stimulating factor and interleukin-3 on eosinophil lineage. *Int. J. Hematol.* 58:21-26.

166. Scott, R.E., and R.G. Horn. 1970. Fine structural features of eosinophilic granulocyte development in human bone marrow. *J. Ultrastruct. Res.* 33:16-28.

167. Dvorak, A.M., and T. Ishizaka. 1994. Human eosinophils *in vitro*. An ultrastructural morphology primer. *Histol. Histopathol.* 9:339-374.

168. Popken-Harris P., J. Checkel, D. Loegering, B. Madden, M. Springett, G. Kephart, and G.J. Gleich. 1998. Regulation and processing of a precursor form of eosinophil granule major basic protein (ProMBP) in differentiating eosinophils. *Blood.* 92:623-631.

169. Sehmi, R, L.J. Wood, R. Watson, R. Foley, and P.M. O'Byrne. 1999. An inhaled corticosteroid, budesonide, reduces baseline but not allergen induced increases in bone marrow inflammatory cell progenitors in asthmatic subjects. *Am. J. Respir. Crit. Care Med.*159:1457-1463.

170. Inman, M.D., J.A. Denburg, R. Ellis, M. Dahlback, and P.M. O'Byrne. 1997. The effect of treatment with budesonide or PGE2 *in vitro* on allergen induced increases in canine bone marrow progenitors. *Am. J. Respir. Cell. Mol. Biol.* 17:634-641.

171. Lewis, D.M., I.C. Lewis, D.A. Loegering, and G.J. Gleich. 1978. Localization of the guinea pig eosinophil major eosinophil basic protein to the core of the granule. *J. Cell. Biol.* 77:702-713.

172. Plager D.A., S. Stuart and G.J. Gleich. 1998. Human eosinophil granule major basic protein and its novel homolog. *Allergy.* 53: 33-40.

173. Wasmoen, T.L., M.P. Bell, D.A. Loegering, G.J. Gleich, F.G. Prendergast, and D.J. McKean. 1988. Biochemical and amino acid sequence analysis of human eosinophil granule major basic protein. *J. Biol. Chem.* 263:12559-12563.

174. Kita, H., R.I. Abu Ghazaleh, S. Sur, and G.J. Gleich. 1995. Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. *J. Immunol.* 154:4749-4758.

175. Kroegel, C., U. Costabel, and H. Matthys. 1987. Mechanism of membrane damage mediated by eosinophil major basic protein. *Lancet.* 1:1380-1381.

176. Egesten, A., J. Alumets, C. von Mecklenburg, M. Palmegren, and I. Olsson. 1986. Localization of eosinophil cationic protein, major basic protein, and eosinophil peroxidase in human eosinophils by immunoelectron microscopic technique. *J. Histochem. Cytochem.* 34:1399-403.

177. Ackerman, S.J., D.A. Loegering, P. Venge, I. Olsson, J.B. Harley, A.S. Fauci, and G.H. Gleich. 1983. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* 131:2977-2982.

178. Watanabe, M., T. Nittoh, T. Suzuki, A. Kitoh, S. Mue, and K. Ohuchi. 1995. Isolation and partial characterization of eosinophil granule proteins in rats -- eosinophil cationic protein and major basic protein. *Int. Arch. Allergy Immunol.* 108:11-18.

179. Gleich, G.J., D.A. Loegering, M.P. Bell, J.L. Checkel, S.J. Ackerman, and D.J. McKean. 1986. Biochemical and functional similarities between human eosinophil-derived neurotoxin and eosinophil cationic protein: Homology with ribonuclease. *Proc. Natl. Acad. Sci. USA.* 83:3146-3150.

180. Lehrer, R.I., D. Szklarek, A. Barton, T. Ganz, K.J. Hamann, and G.J. Gleich. 1989. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. *J. Immunol.* 142:4428-4434.

181. Young, J.D., C.G. Peterson, P. Venge, and Z.A. Cohn. 1986. Mechanism of membrane damage mediated by human eosinophil cationic protein. *Nature.* 321:613-616.

182. Rosenberg, H.F., K.D. Dyer, H.L. Tiffany, and M. Gonzalez. 1995. Rapid evolution of a unique family of primate ribonuclease genes. *Nat. Genet.* 10:219-223.

183. Rosenberg, H.F., D.G. Tenen, and S.J. Ackerman. 1989. Molecular cloning of the human eosinophil-derived neurotoxin: a member of the ribonuclease gene family. *Proc. Natl. Acad. Sci. USA.* 86: 4460-4464.

184. Barker, R.L., D.A. Loegering, R.M. Ten, K.J. Hamann, L.R. Pease, and G.J. Gleich. 1989. Eosinophil cationic protein cDNA. Comparison with other toxic cationic proteins and ribonucleases. *J. Immunol.* 143:952-955.

185. Durack, D.T., S.M. Sumi, and S.J. Klebanoff. 1979. Neurotoxicity of human eosinophils. *Pro.c Natl. Acad. Sci. USA.* 76:1443-1447.

186. Sorrentino, S., D.G. Glitz, K.J. Hamann, D.A. Loegering, J.L. Checkel, and G.J. Gleich. 1992. Eosinophil-derived neurotoxin and human liver ribonuclease. Identity of structure and linkage of neurotoxicity to nuclease activity. *J. Biol. Chem.* 267:14859-14865.

187. Olsson, I., A.M. Persson, K. Stromberg, I. Winqvist, P.C. Tai, and C.J. Spry. 1985. Purification of eosinophil peroxidase and studies of biosynthesis and processing in human marrow cells. *Blood.* 66:1143-1148.

188. Ten, R.M., L.R. Pease, D.J. McKean, M.P. Bell, and G.J. Gleich. 1989. Molecular cloning of the human eosinophil peroxidase. Evidence for the existence of a peroxidase multigene family. *J. Exp. Med.* 169:1757-1769.

189. Henderson, W.R., A. Jorg, and S.J. Klebanoff. 1982. Eosinophil peroxidase-mediated inactivation of leukotrienes B_4, C_4, and D_4. *J. Immuno.l* 128:2609-2613.

190. Jong, E.C., W.R. Henderson, and S.J. Klebanoff. 1980. Bactericidal activity of eosinophil peroxidase. *J. Immunol.* 124:1378-1382.

191. Schmidt, D., and K.F. Rabe. 2000. The role of leukotrienes in the regulation of tone and responsiveness in isolated human airways. *Am. J. Respir. Crit. Care Med.* 161:S62-67.

192. Bozza, P.T., W. Yu, and P.F. Weller. 1997. Mechanisms of formation and function of eosinophil lipid bodies: inducible intracellular sites involved in arachidonic acid metabolism. *Mem. Inst. Oswaldo Cruz.* 92:135-40.

193. Chung, K.F., and P.J. Barnes. 1991. Role for platelet-activating factor in asthma. *Lipids.* 26:1277-9.

194. Holtzman, M.J. 1991. Arachidonic acid metabolism. Implications of biological chemistry for lung function and disease. *Am. Rev. Respir. Dis.* 143:188-203.

195. Chilton, F.H., J.M. Ellis, S.C. Olson, and R.L. Wykle. 1984. 1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine. A common source of platelet-activating factor and arachidonate in human polymorphonuclear leukocytes. *J. Biol. Chem.* 259:12014-12019.

196. Moqbel, R.1994. Eosinophils, cytokines, and allergic inflammation. *Ann. N. Y. Acad. Sci.* 725:223-233.

197. Lacy, P., and R. Moqbel. 1997. Eokines: synthesis, storage and release from human eosinophils. *Mem. Inst. Oswaldo Cruz.* 92:125-133.

198. Weller, P.F., T.H. Rand, T. Barrett, A. Elovic, D.T.W. Wong, and R.W. Finberg. 1993. Accessory cell function of human eosinophils: HLA-DR–dependent. MHC-restriction antigen presentation and interleukin-1α formation. *J. Immunol.* 150:2554-2562.

199. Bosse, M., M. Audette, C. Ferland, G. Pelletier, H.W. Chu, A. Dakhama, S. Lavigne, L.P. Boulet, and M. Laviolette. 1996. Gene expression of interleukin-2 in purified human peripheral blood eosinophils. *Immunology.* 87:149-154.

200. Kita, H., T. Ohnishi, Y. Okubo, D. Weiler, J.S. Abrams, and G.J. Gleich. 1991. GM-CSF and Interleukin 3 release from human peripheral blood eosinophils and neutrophils. *J. Exp. Med.* 174:743-748.

201. Moqbel, R., S. Ying, J. Barkans, T.M. Newman, P. Kimmitt, M. Wakelin, L. Taborda Barata, Q. Meng, C.J. Corrigan, S.R. Durham, and A.B. Kay. 1995. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *J. Immunol.* 155: 4939-4947.

202. Desreumaux, P.A., A. Janin, J.F. Colombel, L. Prin, J. Plumas, D. Emilie, G. Torpier, A. Capron, and M. Capron. 1992. Interleukin-5 mRNA expression by eosinophils in the intestinal mucosa of patients with coeliac disease. *J. Exp. Med.* 175:293-296.

203. Lacy, P., F. Levi Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J. Velazquez, and A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferonγ. *Blood.* 91:2508-2516.

204. Gounni, A.S., E. Nutku, L. Koussih, F. Aris, J. Louahed, R.C. Levitt, N.C. Nicolaides, and Q. Hamid. 2000. IL-9 expression by human eosinophils: regulation by IL-1beta and TNF-alpha. *J. Allergy Clin. Immunol.* 106:460-466.

205. Nakajima, H., G.J. Gleich, and H. Kita. 1996. Constitutive production of IL-4 and IL-10 and stimulated production of IL-8 by normal peripheral blood eosinophils. *J. Immunol.* 156:4859-4866.

206. Lim, K.G., H.C. Wan, P.T. Bozza, M.B. Resnick, D.T. Wong, W.W. Cruikshank, H. Kornfeld, D.M. Center, and P.F. Weller. 1996. Human eosinophils elaborate the lymphocyte chemoattractants IL-16 (lymphocyte chemoattractant factor) and RANTES. *J. Immunol.* 156:2566-2570.

207. Moqbel, R., Q. Hamid, S. Ying, J. Barkans, A. Hartnell, A. Tsicopoulos, A.J. Wardlaw, and A.B. Kay. 1991. Expression of mRNA and immunoreactivity for the granulocyte/macrophage-colony stimulating factor (GM-CSF) in activated human eosinophils. *J. Exp. Med.* 174:749-52.

208. Nakajima, T., H. Yamada, M. Iikura, M. Miyamasu, S. Izumi, H. Shida, K. Ohta, T. Imai, O. Yoshie, M. Mochizuki, J.M. Schroder, Y. Morita, K. Yamamoto, and K. Hirai. 1998. Intracellular localization and release of eotaxin from normal eosinophils. *FEBS Lett.* 434:226-230.

209. Costa, J.J., K. Matossian, M.B. Resnick, W.J. Beil, D.T. Wong, J.R. Gordon, A.M. Dvorak, P.F. Weller, and S.J. Galli. 1993. Human eosinophils can express the cytokines tumor necrosis factor-α and macrophage inflammatory protein-1α. *J. Clin. Invest.* 91:2673-2684.

210. Ying, S., Q. Meng, L. Taborda Barata, C.J. Corrigan, J. Barkans, B. Assoufi, R. Moqbel, S.R. Durham, and A.B. Kay. 1996. Human eosinophils express messenger RNA encoding RANTES and store and release biologically active RANTES protein. *Eur. J. Immunol.* 26:70-76.

211. Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med.* 179:881-887.

212. Schall, T.J., K. Bacon, K.J. Toy, and D.V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature.* 347:669-671.

213. Taub, D.D., K. Conlon, A.R. Lloyd, J.J. Oppenheim, and D.J. Kelvin. 1993. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. *Science*. 260:355-358.

214. Rot, A., M. Krieger, T. Brunner, S.C. Bischoff, T.J. Schall, and C.A. Dahinden. 1992. RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J. Exp. Med.* 176:1489-1495.

215. Alam, R., S. Stafford, P. Forsythe, R. Harrison, D. Faubion, M.A. Lett-Brown, and J.A. Grant. 1993. RANTES is a chemotactic and activating factor for human eosinophils. *J. Immunol.* 150:3442-3448.

216. Kapp, A., G. Zeck-Kapp, W. Czech, and E. Schopf. 1994. The chemokine RANTES is more than a chemoattractant: characterization of its effect on human

eosinophil oxidative metabolism and morphology in comparison with IL-5 and GM-CSF. *J. Invest. Dermatol.* 102:906-914.

217. Lamkhioued, B., A.S. Gounni, D. Aldebert, E. Delaporte, L. Prin, A. Capron, M. Capron. 1996. Synthesis of type 1 (IFN gamma) and type 2 (IL-4, IL-5, and IL-10) cytokines by human eosinophils. *Ann. N. Y. Acad. Sci.* 796:203-208.

218. Kita, H., T. Ohnishi, Y. Okubo, D. Weiler, J.S. Abrams, and G.J. Gleich. 1991. Granulocyte/macrophage colony-stimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. *J. Exp. Med.* 174:745-748.

219. Wong, D.T., P.F. Weller, S.J. Galli, A. Elovic, T.H. Rand, G.T. Gallagher, T. Chiang, M.Y. Chou, K. Matossian, J. McBride, and R. Todd. 1990. Human eosinophils express transforming growth factor alpha. *J. Exp. Med.* 172:673-681.

220. Tan, X., W. Hsueh, and F. Gonzalez-Crussi. 1991. Cellular localization of tumor necrosis factor (TNF)-alpha transcripts in normal bowel and in necrotizing enterocolitis. TNF gene expression by Paneth cells, intestinal eosinophils, and macrophages. *Am. J. Pathol.* 142:1858-1865.

221. Powell, P.P., M. Klagsbrun, J.A. Abraham, and R.C. Jones. 1993. Eosinophils expressing heparin-binding EGF-like growth factor mRNA localize around lung microvessels in pulmonary hypertension. *Am. J. Pathol.* 143:784-793.

222. Solomon, A., L. Aloe, J. Pe'er, J. Frucht-Pery, S. Bonini, S. Bonini, and F. Levi-Schaffer. 1998. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *J. Allergy Clin. Immunol.* 102:454-460.

223. Hartman, M., A. Piliponsky, V. Temkin, and F. Levi-Schaffer. 1999. Human peripheral blood eosinophils as a novel source of stem cell factor. (abstr 7000). *J. Allergy Clin. Immunol.* 103:S183.

224. Persson, C.G., and J.S. Erjefalt. 1997. Eosinophil lysis and free granules: an *in vivo* paradigm for cell activation and drug development. *Trends Pharmacol. Sci.* 18:117-123.

225. Leiferman, K.M., T. Fujisawa, B.H. Gray, and G.J. Gleich. 1990. Extracellular deposition of eosinophil and neutrophil granule proteins in the IgE-mediated cutaneous late phase reaction. *Lab. Invest*. 62:579-589.

226. Dvorak, A.M., P.F. Weller, R.A. Monahan-Earley, L. Letourneau, and S.J. Ackerman. 1990. Ultrastructural localization of Charcot-Leyden crystal protein (lysophospholipase) and peroxidase in macrophages, eosinophils, and extracellular matrix of the skin in the hypereosinophilic syndrome. *Lab. Invest*. 62:590-607.

227. Dvorak, A.M., M.C. Mihm, J.E. Osage, T.H. Kwan, K.F. Austen, and B.U. Wintroub. 1982. Bullous pemphigoid, an ultrastructural study of the inflammatory response: eosinophil, basophil and mast cell granule changes in multiple biopsies from one patient. *J. Invest. Dermatol.* 78:91-101.

228. Scepek, S., and M. Lindau. 1993. Focal exocytosis by eosinophils--compound exocytosis and cumulative fusion. *EMBO J.* 12:1811-1817.

229. Erjefalt, J.S., M. Andersson, L. Greiff, M. Korsgren, M. Gizycki, P.K. Jeffery, and G.A. Persson. 1998. Cytolysis and PMD as distinct modes of activation of airway mucosal eosinophils. *J. Allergy Clin. Immunol.* 102:286-294.

230. Moqbel, R., and P. Lacy. 1999. Exocytotic events in eosinophils and mast cells. *Cli.n Exp. Allergy.* 29:1017-1022.

231. Dvorak, A.M., T. Furitsu, L. Letourneau, T. Ishizaka, and S.J. Ackerman. 1991. Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part 1. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. *Am. J. Pathol.* 138:69-82.

232. Capron, M., M. Tomassini, G. Torpier, J.P. Kusnierz, S. MacDonald, and A. Capron. 1989. Selectivity of mediators released by eosinophils. *Int. Arch. Allergy Appl. Immunol.* 88:54-58.

233. Tomassini, M., A. Tsicopoulos, P.C. Tai, V. Gruart, A.B. Tonnel, L. Prin, A. Capron, and M. Capron. 1991. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J. Allergy Clin. Immunol.* 88:365-375.

234. Sollner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J.E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature.* 362:318-324.

235. Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, dL. Polverino, B.R. DasGupta, and C. Montecucco. 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature.* 359:832-835.

236. Schiavo, G., M. Matteoli, and C. Montecucco. 2000. Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* 80:717-766.

237. Beckers, C.J., M.R. Block, B.S. Glick, J.E. Rothman, and W.E. Balch. 1989. Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature.* 339:397-398.

238. Hay, J.C., and R.H. Scheller. 1997. SNAREs and NSF in targeted membrane fusion. *Curr.Opin.Cell Biol.* 9:505-512.

239. Whiteheart, S.W., M. Brunner, D.W. Wilson, M. Wiedmann, and J.E. Rothman. 1992. Soluble N-ethylmaleimide-sensitive fusion attachment proteins (SNAPs) bind to a multi-SNAP receptor complex in Golgi membranes. *J. Biol.Chem.* 267:12239-12243.

240. Ravichandran, V., A. Chawla, and P.A. Roche. 1996. Identification of a novel syntaxin- and synaptobrevin/VAMP- binding protein, SNAP-23, expressed in non-neuronal tissues. *J. Biol. Chem.* 27:13300-13303.

241. Kawanishi, M., Y. Tamori, H. Okazawa, S. Araki, H. Shinoda, and M. Kasuga. 2000. Role of SNAP23 in insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. *J. Biol.Chem.* 275:8240-8247.

242. Foster, L.J., K. Yaworsky, W.S. Trimble, and A. Klip. 1999. SNAP23 promotes insulin-dependent glucose uptake in 3T3-L1 adipocytes: possible interaction with cytoskeleton. *Am. J. Physiol.* 276:C1108-C1114.

243. Guo, Z., C. Turner, and D. Castle. 1998. Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell.* 94:537-548.

244. Paumet, F., J. Le Mao, S. Martin, T. Galli, B. David, U. Blank, and M. Roa. 2000. Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. *J. Immunol.* 164:5850-5857.

245. Flaumenhaft, R., K. Croce, E. Chen, B. Furie, and B.C. Furie. 1999. Proteins of the exocytotic core complex mediate platelet a-granule secretion. Roles of vesicle-associated membrane protein, SNAP-23, and syntaxin 4. *J.Biol.Chem.* 274:2492-2501.

246. Chen, D., A.M. Bernstein, P.P. Lemons, and S.W. Whiteheart. 2000. Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. *Blood.* 95:921-929.

247. Lemons, P.P., D. Chen, A.M. Bernstein, M.K. Bennett, and S.W. Whiteheart. 1997. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood.* 90:1490-1500.

248. Ho, J.L., and M.S. Klempner. 1985. Tetanus toxin inhibits secretion of lysosomal contents from human macrophages. *J. Infect.Dis.* 152:922-929.

249. Maecker, H.T., S.C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J.* 11:428-442.

250. Wright M.D., and M.G. Tomlinson MG. 1994. The ins and outs of the transmembrane 4 superfamily. *Immunol. Today,* 15:588-594.

251. Berditchevski, F., K.F. Tolias, K. Wong, C.L. Carpenter, and M.E. Hemler. 1997. A novel link between integrins, TM4SF proteins (CD63, CD81) and phosphatidylinositol 4-kinase. *J. Biol. Chem.* 272:2595–2598.

252. Sugiura, T. and F. Berditchevski. 1999. Function of $\alpha3\beta1$-tetraspanin protein complexes in tumor cell invasion. Evidence for the role of the complexes in production of matrix metalloproteinase 2 (MMP-2). *J. Cell Biol.* 146:1375–1389.

253. Schwartz-Albiez, R., B. Dorken, W. Hofmann, and G. Moldenhauer. 1988. The B cell-associated CD37 antigen (gp40–52). Structure and subcellular expression of an extensively glycosylated glycoprotein. *J. Immunol.* 140:905–914.

254. Berger, G., J.M. Masse, and E.M. Cramer. 1996. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood.* 87:1385–1395.

255. Cramer, E. M., G. Berger, and M. Berndt. 1994. Platelet alpha-granule and plasma membrane share two new components: CD9 and PECAM-1. *Blood.* 84:1722–1730.

256. Escola, J. M., M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, and H.J. Geuze, H. J. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273:20121–20127.

257. Hammond, C., L.K. Denzin, M. Pan, J.M. Griffith, H.J. Geuze, and P. Cresswell. 1998. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J. Immunol.* 161:3282–3291.

258. Sincock, P. M., S. Fitter, R.G. Parton, M.C. Berndt, J.R. Gamble, and L.K. Ashman. 1999. PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function. *J. Cell Sci.* 112:833–844.

259. Altman, J. 1988. Ins and outs of cell signalling. *Nature,* 331:119-20.

260. Berditchevski, F., and E. Odintsova. 1999. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J. Cell. Biol.* 146:477-492.

261. Rubinstein, E., F. Le Naour, C. Lagaudriere-Gesbert, M. Billard, H. Conjeaud, and C. Boucheix. 1996. CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur. J. Immunol.* 26:2657-2665.

262. Higginbottom, A., I. Wilkinson, B. McCullough, F. Lanza, D.O. Azorsa, L.J. Partridge, and P.N. Monk. 2000. Antibody cross-linking of human CD9 and the high-affinity immunoglobulin E receptor stimulates secretion from transfected rat basophilic leukaemia cells. *Immunology.* 99:546-452.

263. Kobayashi, T., U.M. Vischer, C. Rosnoblet, C. Lebrand, M. Lindsay, R.G. Parton, E.K. Kruithof, and J. Gruenberg. 2000. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Mol.Biol.Cell* 11:1829-1843.

264. Fukuda, M.1991. Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol.Chem.* 266:21327-21330.

265. Nieuwenhuis, H.K., J.J. van Oosterhout, E. Rozemuller, F. van Iwaarden, and J.J.Sixma. 1987. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood.* 70:838-845.

266. Moneret-Vautrin, D.A., J. Sainte-Laudy, G. Kanny, and S. Fremont. 1999. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. *Ann. Allergy. Asthma. Immunol.* 82:33-40.

267. Viksman, M.Y., M.C. Liu, C.A. Bickel, R.P. Schleimer, and B.S. Bochner. 1997. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. I. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. *Am. J. Respir. Crit. Care Med.* 155:858-863.

268. Skubitz, K.M., K.D. Campbell, J. Iida, A.P. Skubitz. 1996. CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. *J. Immunol.* 157:3617-3626.

269. Metzelaar, M. J., P.L.J. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, and H.C. Clevers. 1991. CD63 antigen: a novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* 266:3239–3245.

270. Egesten, A., J. Calafat, E.F. Knol, H. Janssen, and T.M. Walz. 1996. Subcellular localization of transforming growth factor-alpha in human eosinophil granulocytes. *Blood.* 87:3910-3918.

271. Mengelers, H.J., T. Maikoe, L. Brinkman, B. Hooibrink, J.W. Lammers, and L. Koenderman. 1994. Immunophenotyping of eosinophils recovered from blood and BAL of allergic asthmatics. *Am. J. Respir. Crit. Care Med.* 149:345-351.

272. Koyama, Y., M. Suzuki, T. Yoshida. 1998. CD63, a member of tetraspan transmembrane protein family, induces cellular spreading by reaction with monoclonal antibody on substrata. Biochem. *Biophys. Res. Commun.* 246:841-846.

273. Vischer, U.M., and D.D. Wagner. 1993. CD63 is a component of Weibel-Palade bodies of human endothelial cells. *Blood.* 82:1184-1191.

274. Toothill, V.J., J.A. Van Mourik, H.K. Niewenhuis, M.J. Metzelaar, and J.D. Pearson. 1990. Characterization of the enhanced adhesion of neutrophil leukocytes to thrombin-stimulated endothelial cells. *J. Immunol.* 145:283-291.

275. Johansson, A., R. Claesson, L. Hanstrom, G. Sandstrom, and S. Kalfas. 2000. Polymorphonuclear leukocyte degranulation induced by leukotoxin from Actinobacillus actinomycetemcomitans. *J. Periodontal. Res.* 35:85-92.

276. Atkinson, B., C.S. Ernst, B.F. Ghrist, A.H. Ross, W.H. Clark, M. Herlyn, D. Herlyn, G. Maul, and Z. Steplewski and H. Koprowski. 1985. Monoclonal antibody to a highly glycosylated protein reacts in fixed tissue with melanoma and other tumors. *Hybridoma* 4:243–255.

277. Kondoh, M., M. Ueda, M. Ichihashi, and Y. Mishima. 1993. Decreased expression of human melanoma-associated antigen ME491 along the progression of melanoma pre-canceroses to invasive and metastatic melanomas. *Melanoma Res.* 3:241–245.

278. Hotta, H., H. Miyamoto, L. Hara, N. Takahashi, and M. Homma. 1992. Genomic structure of the ME491/CD63 antigen gene and functional analysis of the 5'-flanking regulatory sequences. *Biochem. Biophys. Res. Com.* 185:436–442.

279. Anton, E. S., M. Hadjiargyrou, P.H. Patterson, and W.D. Matthew. 1995. CD9 plays a role in Schwann cell migration in vitro. *J. Neuroscience* 15:584–595.

280. Radford, K.J., R.F. Thorne, P. Hersey. 1996. CD63 associates with transmembrane 4 superfamily members, CD9 and CD81, and with beta 1 integrins in human melanoma. *Biochem. Biophys. Res. Commun.* 222:13-18.

281. Yauch R.L. and E.M. Hemler. 2000. Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem. J.* 351:629–637.

282. Knol, E.F., F.P. Mul, H. Jansen, J. Calafat, and D. Roos. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J. Allergy. Clin.Immunol.* 88:328-338.

283. Smith, D.A., P.N. Monk, and L.J. Partridge. 1995. Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells. *Mol. Immunol.* 32:1339-1344.

284. Tapper, H., and S. Grinstein. 1997. Fc receptor-triggered insertion of secretory granules into the plasma membrane of human neutrophils: selective retrieval during phagocytosis. *J. Immunol.* 159:409-418.

285. Israels S.J., E.M. McMillan-Ward, J. Easton, C. Robertson, and A. McNicol. 2001. CD63 associates with the alphaIIb beta3 integrin-CD9 complex on the surface of activated platelets. *Thromb. Haemost.* 85:134-41.

Chapter 2

Immunofluorescence analysis of cytokine and granule protein expression during eosinophil maturation from cord blood-derived CD34$^+$ progenitors[1]

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[1] Authors, S. Mahmudi-Azer, J. Velazquez, P. Lacy, B. Bablitz, and R. Moqbel. A version of this chapter was published in *J. Allergy Clin. Immunol.* 2000 105:1178-84. I contributed to this work by carrying out the majority of the experimental procedures, including cytospin preparation, immunofluorescent staining and confocal laser scanning microscopy, and also by preparing and writing the manuscript.

Introduction

Eosinophils are prominent cells in allergic inflammation, asthma and host defense against parasitic diseases (1-4). These inflammatory leukocytes are apparently derived from myelocytic progenitors in the bone marrow (5,6), which have been shown to be $CD34^+$ (7,8). Eosinophils store and release, upon appropriate activation, a wide spectrum of pro-inflammatory mediators including cationic granule proteins; major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (1, 2). They have also been shown to synthesize up to 23 cytokines, chemokines and growth factors, many of which are stored in their crystalloid granules (9,10). Ultrastructurally, the crystalloid granule is comprised of two compartments, namely an electron-dense crystalline core (internum), and an electron-lucent matrix. Mediators have been shown to be differentially stored within these two compartments; thus while MBP (11,12), GM-CSF (13), and IL-2, (14), have been detected in the internum, ECP, EDN, EPO (1,11), together with a number of other cytokines and chemokines including IL-5 (15), IL-6 (16), RANTES (17) and TNFα (18) appear to be stored within the granule matrix.

Recombinant human interleukin 3 (rhIL-3) and interleukin 5 (rhIL-5) are known to induce the differentiation of maturing eosinophils from isolated progenitors in *in vitro* culture systems (19, 20). Indeed, the morphological characteristics of eosinophilopoiesis and the ontogeny of the formation of crystalloid granule have been partially investigated by analysis of eosinophil progenitors using electron microscopy and *in vitro* culture system (21). This included the description of early commitment to the eosinophil phenotype by the appearance of numerous large immature granules in the cytoplasm (21). The observation that immature granules appear during early eosinophil development was confirmed in later studies showing pro-MBP mRNA and protein expression in developing eosinophils *in vitro,* which localized to large immature granules containing "hollow" electron-lucent cores (22). None of these studies, however, have examined the expression and pattern of granule storage of cytokines, in parallel with cationic proteins, in differentiating eosinophils.

We have recently demonstrated the transcription of cytokines (IL-4 and RANTES) in maturing eosinophils cultured from CD34$^+$ progenitors (23). In this study, using *in vitro* culture system and confocal laser scanning microscopy, we aimed to investigate the production of IL-6 and RANTES at the protein level, during rhIL-3 and rhIL-5-induced eosinophil maturation from CD34$^+$ progenitors, and their ultimate storage in crystalloid granule-like structures. Our previous studies have shown that confocal laser scanning microscopy (CLSM) is a powerful technique in detecting cytokine expression and storage in peripheral blood eosinophils (16,17). MBP and ECP were chosen as markers of eosinophil crystalloid granules, while IL-6 and RANTES were representative of eosinophil-derived cytokines and chemokines, respectively.

Our data describe the relationship between the storage patterns of cytokines juxtaposed with cationic granule proteins during the course of eosinophil maturation. This study provides new insights on mediator expression during *in vitro* eosinophilopoiesis. In addition, for the first time, our study shows that cytokine storage is an early event in the natural history of the eosinophil and not only confined to mature or activated cells.

Methods

Isolation and purification of CD34$^+$ cells from cord blood

Human cord blood samples were provided by Dr. B. H. Mitchell, Prenatal Research Center, Royal Alexandra Hospital, Edmonton, AB, Canada. A total of 25 mL of cord blood was diluted 1:4 in PBS and loaded onto a 45-mL Ficoll-Paque (Pharmacia Biotech, Inc, Uppsala, Sweden) followed by centrifugation at 400 *g* at room temperature for 25 min. The upper layer containing mononuclear cells was collected and washed twice in 5 mM PBS EDTA. The isolated mononuclear layer was incubated with micromagnetic beads coupled to anti-CD34 mAb (QBEND-10) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 30 min, and subjected to positive selection by loading the cell suspension on a Mini-MACS separation column (Miltenyi Biotec). CD34$^+$ cells were eluted by positive selection after removing the column from the magnetic field. The purity of isolated cells (>95%) was determined using immunofluorescent labeling with phycoerythrin-conjugated antibody (anti-HPCA-2; Becton Dickinson, Mississauga, ON, Canada) and FACS analysis. The number and viability (>99%) of purified CD34$^+$ cells was determined by trypan blue exclusion.

Semi-solid culture of CD34$^+$ cells

Purified CD34$^+$ cells were cultured in 35mm Petri dishes (Falcon Plastic, Oxnard, CA) at a concentration of 4×10^4 cells/ml in RPMI supplemented with Iscove's Modified Dulbecco's (BioWhittaker, Walkersville, MD), β-mercaptoethanol (1×10^{-5} M), penicillin

(10,000 units/ml), streptomycin (10 mg/ml), 0.3% $NaHCO_3$, 20% heat-inactivated FCS (Gibco BRL Life Technologies, Ltd, Burlington, ON, Canada), 5 ng/ml rhIL-3 (Genzyme, Markham, ON, Canada), 2 ng/ml rhIL-5 (Pharmingen, Mississauga, ON, Canada), and 0.84% methylcellulose (StemCell Technologies Inc, Vancouver, BC, Canada). Cultured cells were maintained at 37 °C in 5% CO_2, examined at weekly intervals and the cytokine concentrations adjusted.

Cell collection and cytospin preparation

Cytospins of freshly purified $CD34^+$ cells and *in vitro* cultured cells at days 16, 19, 23 and 28, were prepared by spinning $2x10^4$ cells suspended in 100 µl 20% FCS in RPMI-1640 in a Cytospin 2 centrifuge (Shandon Ltd, Runcorn, UK) at 800 rpm for 2 minutes. Cytospins were air-dried and stored at -20°C until used.

Immunofluorescent labeling (CD34, MBP, ECP, IL-6 and RANTES)

Slides of purified $CD34^+$ and cultured cells (days 16, 19, 23, 28) were fixed for 8 min in 2% paraformaldehyde in PBS (room temperature) and washed five times in Tris-buffered saline (TBS, pH 7.4). Following fixation, cells were blocked using 3% FCS in a humidified container for 30 min. After a second washing step, slides were incubated overnight with TBS containing 1% mouse monoclonal anti-human MBP (BMK-13, generated in-house) at 4°C. Immunoreactivity to MBP was detected by incubating slides with15 µg/mL Rhodamine (TRITC)-labeled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for 2 h. Following another washing step, slides were blocked again for 2 h using 50 µg/mL goat anti-mouse IgG fab fragment (Molecular Probes, Eugene, OR) and double-labeled with one of the following: primary mouse monoclonal anti-human ECP (EG2), (2 μg/mL, Kabi Pharmacia, Newington, NH), anti-human IL-6 (2 μg/mL) (R&D Systems, Minneapolis, MI), anti-human RANTES (5 μg/mL) (R&D Systems) overnight at 4°C. Immunoreactivity against ECP, IL-6 and RANTES were visualized using 20 μg/mL BODIPY FL-conjugated goat anti-mouse antibody (Molecular Probes). In double immunofluorescent staining of cytospins of purified CD34$^+$ cells, phycoerythrin-conjugated anti-CD34 (10 μg/mL) (Becton Dickinson) was used to detect CD34 expression, and BODIPY-FL conjugated secondary antibody (20 μg/mL) was used to detect immunoreactivity for MBP, ECP, IL-6 and RANTES. Mouse IgG1 (R&D Systems) at equivalent concentrations was used as the isotype control. After a final washing step, 10 μl of the antibleaching agent, (0.4% n-propyl gallate (Sigma, Oakville, ON, Canada) in 3:1 glycerol: 10xTBS) was applied to the slides before coverslip attachment.

Confocal laser scanning microscopy

Immunofluorescent staining of freshly purified CD34$^+$ and developing eosinophils was examined using a Leica confocal laser scanning microscope as indicated in earlier reports (16,17).

Results

Immunofluorescence of cytokines in purified cord blood-derived CD34$^+$ cells

We examined the immunoreactivity of freshly prepared CD34$^+$ cells purified from human cord blood within 12 h of collection. Immunofluorescence studies in double-labeled cells showed no detectable immunoreactivity against ECP (Fig. 1 U), MBP (Fig. 1 V), IL-6 (Fig. 1 W) and RANTES (Fig. 1 Z), in cells positive for CD34. Interestingly, although our earlier study (23) indicated that cord blood-derived CD34$^+$ cells express mRNA for RANTES, our immunofluorescent staining suggested that the translated protein for RANTES was undetectable. This was in contrast to CD34$^+$-derived maturing eosinophils produced in culture from day 16 onward, which exhibited positive immunoreactivity to MBP, ECP, IL-6 and RANTES.

Immunofluorescent staining characteristics of maturing eosinophils

To examine the expression and storage of granule cationic proteins (MBP, ECP) together with cytokines (IL-6, RANTES) and to characterize the immunostaining pattern of colonies collected from selected time points, cytospins of cultured cells on days 16, 19, 23 and 28 were prepared and double-labeled with BMK-13 followed by EG_2, anti-human IL-6 or anti-human RANTES. Images taken from isotype controls (Figure 1 Q-T) demonstrate that there was negligible autofluorescence or nonspecific binding in these samples. In these figures, isotype control images were visualized by artificial enhancement of the images.

Immunofluorescent staining of developing eosinophils with anti-MBP and anti-ECP

Analysis of the developing eosinophils immunoreactivity for MBP and ECP at day 16 indicated that most of the cells were mononuclear in appearance with a large nucleus and diffuse immunostaining. As shown in Fig. 1, the pattern of immunoreactivity to MBP and ECP displayed a number of large granule-like structures suggesting that these may localize to immature granules. The majority of these granule-like structures was spherical in appearance, and exhibited intense immunoreactivity against MBP and ECP, which mostly localized to the periphery of these structures (Fig. 1 A-D). At this stage of culture, ECP predominantly co-localized with MBP, which resulted in the appearance of yellow color in the combined image (Fig. 1 C and D). At day 19, maturing eosinophils were mononuclear in appearance and contained a heterogeneous population of granule-like structures with various sizes (Fig. 1 E-H). These structures showed heterogeneous immunostaining with intense immunoreactivity against MBP and ECP localizing to their periphery, and negligible immunoreactivity in the core regions (Fig. 1H). On day 19, MBP and ECP co-localization was not as evident as day 16, and sites of immunoreactivity were observed which roughly coincided with either ECP (green regions in Fig. 1E) or MBP (red regions in Fig. 1F), indicating distinct localization sites for either of the granule cationic proteins (shown in combined images in Fig.1 G and H). At day 23, immunostaining pattern of the developing eosinophils began to approach the immunofluorescent characteristics of mature peripheral blood eosinophils. Developing cells from day 23, immunostained for granular cationic proteins, showed signs of lobular formation in their nuclei (large dark shape) (Fig.1 I-K). Developing eosinophils on day 23 showed strong immunoreactivity for MBP and ECP; however, the large granular structures, which were previously seen at earlier stages of culture, were less visible since immunoreactivity against MBP and ECP appeared to localize to distinct intracellular sites with very little overlap (Fig. 1L). At day 28, the immunoreactivity pattern of cultured cells resembled that of mature peripheral blood eosinophils, in that they exhibited bilobed nuclei and highly focused regions of immunoreactivity to MBP and ECP. Interestingly, the immunostaining pattern of some granular structures was similar to that previously described in mature peripheral blood crystalloid granules (17). Immunoreactivity to ECP appeared to localize to the periphery of granular structures, while immunoreactivity to MBP localized to the core of these structures (Fig. 1P). Nevertheless, unlike mature peripheral blood eosinophils, which contain a large number of crystalloid granules homogeneously distributed in cytoplasm, the number of granular structures observed in cultured cells was substantially less. The reduced number of granular structures in day 28 cultured cells may be due to the release of the granules under these culture conditions because of increased fragility and cell activation (Fig. 1 M-P).

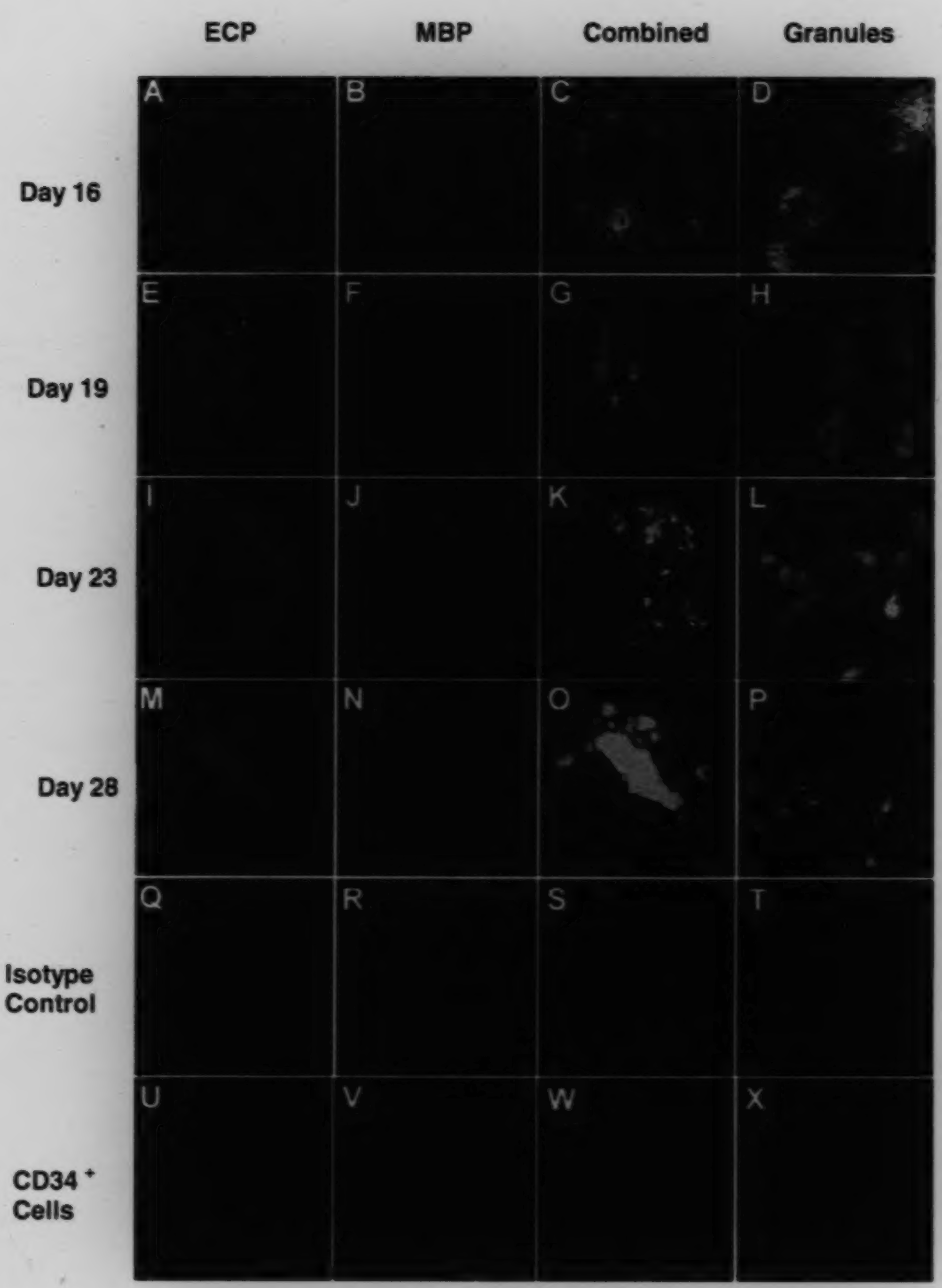
ECP
MBP
Combined
Granules
Day 16
Day 19
Day 23
Day 28
Isotype Control
CD34⁺ Cells
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X

Fig. 2.1. CLSM images of single and double immunofluorescent staining of maturing eosinophils with antibodies for MBP and ECP, freshly isolated cord blood–derived $CD34^+$ progenitor, magnified images of granules and isotype control. The BODIPY-FL channel (green) corresponded to ECP immunoreactivity of developing eosinophils at days 16 (A), 19 (E), 23 (I), and 28 (M) of culture. The rhodamine (TRITC) staining (red) corresponded to MBP at days 16 (B), 19 (F), 23 (J), and 28 (N). Combined images of the same cells are shown to the right (C, G, K, O) and yellow color in these images indicates the colocalization of cationic granule proteins. Isotype control images for ECP, MBP, combined (day 19 of culture), and purified $CD34^+$ are shown in Q through T, respectively. Maturing eosinophils from day 16 and after, unlike freshly isolated cord-blood $CD34^+$ progenitors, exhibited positive immunoreactivity to MBP and ECP. The inset in T shows a freshly isolated $CD34^+$ cell, double-labeled with antibodies for MBP (green) and CD34 (red). *Right,* Magnified images of granules at day 16 (D), 19 (H), 23 (L), and 28 (P). Double-labeled $CD34^+$ cells with specific antibodies against CD34 (Red) with either of ECP (U), MBP (V), IL-6 (W) and RANTES (X). (Original magnification ×100.) Bar (A) indicates 10 μm.

Immunoreactivity of developing eosinophils to anti-IL-6 and RANTES

Developing eosinophils at day 16 of culture co-expressed MBP and IL-6 as well as MBP and RANTES. The immunostaining pattern of day 16 cells with IL-6 and MBP along with RANTES and MBP was comparable to that of MBP and ECP. Immunoreactivity for both IL-6 (Fig. 2) and RANTES (Fig. 3) predominantly co-localized with granule proteins indicating a similar storage pattern for these mediators at early stages of development.

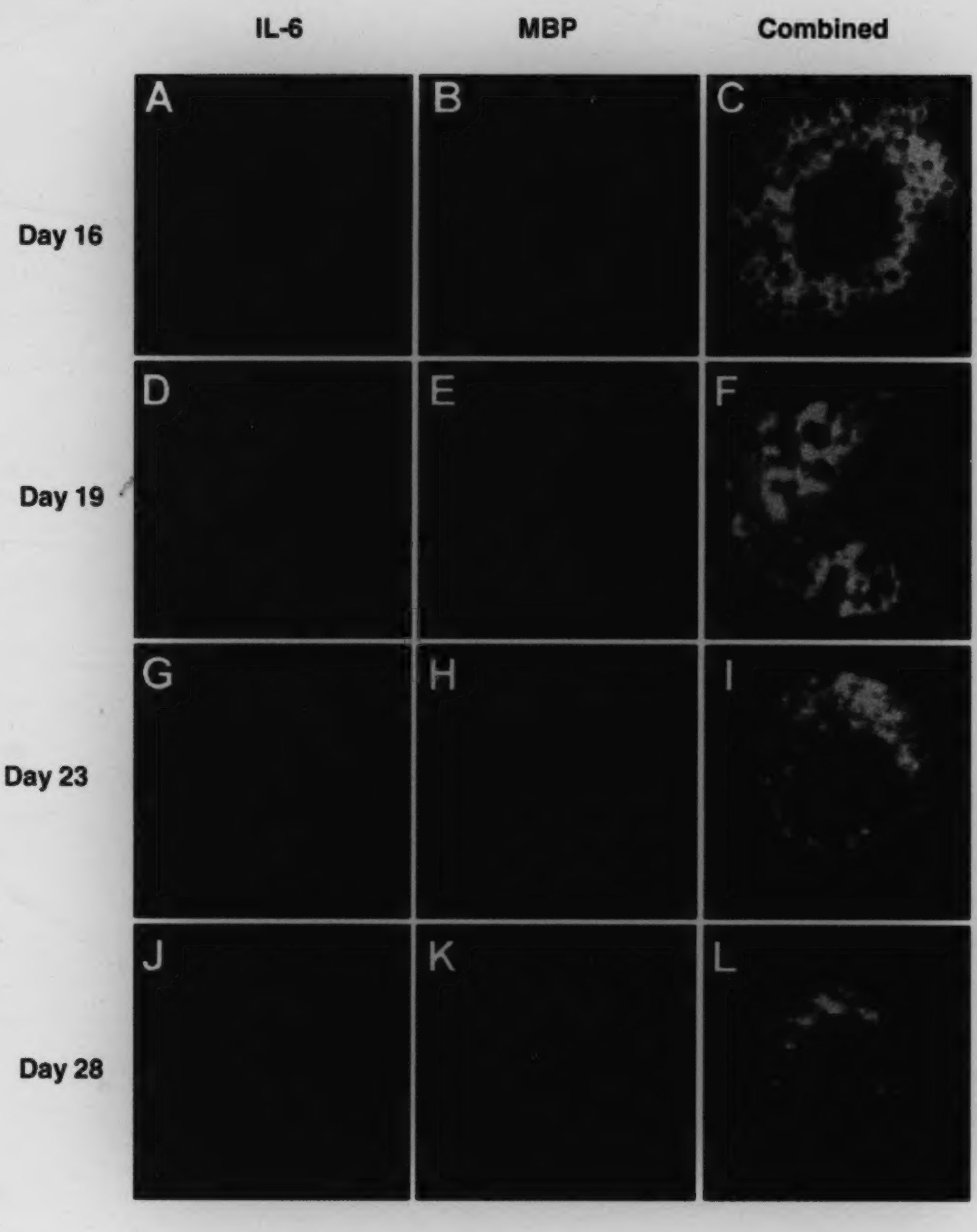
IL-6
MBP
Combined
Day 16
Day 19
Day 23
Day 28
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 2.2 CLSM images of developing eosinophils immunostained for MBP and IL-6. Developing eosinophils were labeled with BODIPY FL (green) representing IL-6 staining at days 16 (A), 19 (D), 23 (G), 28 (J), and TRITC (red) demonstrating MBP staining at days 16 (B), 19 (E), 23 (H), and 28 (K). *Right,* combined MBP and RANTES immunoreactivity in developing eosinophils at days 16 (C), 19 (F), 23 (I), and 28 (L).

At day 19 of culture, immunoreactivity against IL-6 and RANTES were observed to be localized to the granular structures similar to those observed with ECP. However, unlike ECP and MBP, which were distributed in distinct compartments, IL-6 and RANTES co-localized strongly with MBP as indicated by yellow color in the combined images (Fig. 2 and 3 C, F, I, L). By day 23 of culture, immunoreactivity to IL-6 and RANTES in developing eosinophils localized to a population of smaller sized granular structures. On day 28, immunoreactivity to IL-6 and MBP in addition to RANTES and MBP, showed immunostaining patterns in developing eosinophils, which approached that of mature peripheral blood eosinophils. Immunoreactivity to IL-6 and RANTES in day 28 cells localized to a population of granular structures, which exhibited an immunostaining pattern suggestive of the appearance of crystalloid granules. Namely, while immunoreactivity to IL-6 and RANTES localized to the periphery of the granular structures, MBP staining localized to the core regions of these structures (Fig.2 and 3 J-L).

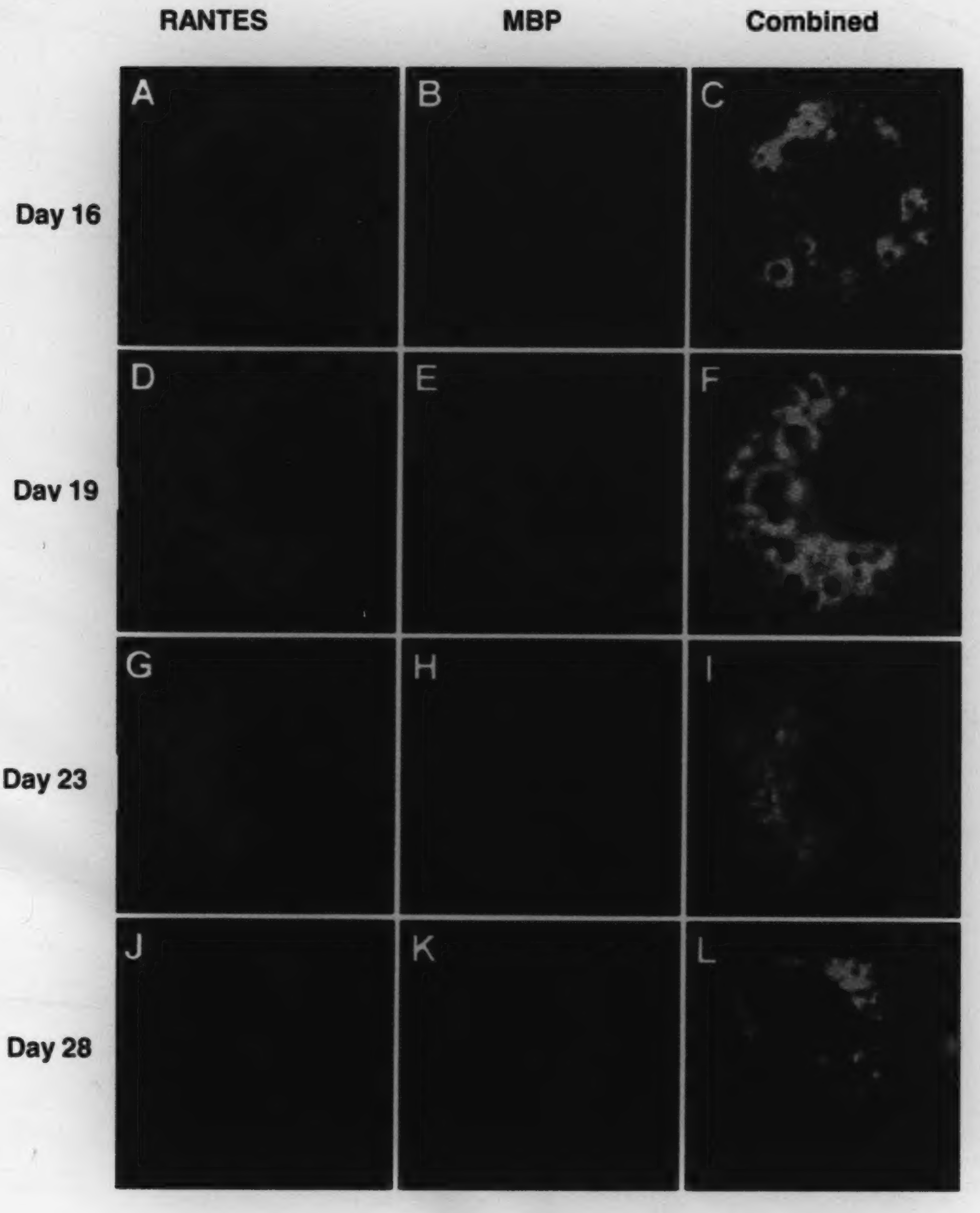
RANTES
MBP
Combined
Day 16
Day 19
Day 23
Day 28
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 2.3. Images of developing eosinophils immunostained for MBP and RANTES. Green color represents RANTES immunostaining in developing eosinophils at days 16 (A), 19 (D), 23 (G), and 28 (L), whereas red color is associated with MBP immunostaining at days 16 (B), 19 (E), 23 (H), and 28 (K). Combined images for each row are shown in (C, F, I, and L).

Discussion

The main objective of our study was to investigate the expression and storage of eosinophil-derived cytokines/chemokines in comparison with granule cationic proteins in cord blood CD34$^+$ cell-derived maturing eosinophils. *In vitro* culture of CD34$^+$ cells in the presence of appropriate cytokines has provided an excellent tool for researchers in the field of hemopoiesis to study leukocyte maturation and differentiation. We have combined this method with double immunofluorescent staining together with CLSM to examine the evolution of the expression pattern of cytokines/chemokines and granule cationic proteins during maturation and differentiation.

We chose to examine the expression and storage pattern of four eosinophil-derived mediators, namely MBP, ECP, IL-6 and RANTES. The intracellular storage patterns of MBP and ECP are well known in circulating and tissue eosinophils (1,11,12).

Although EG_2 (24), was originally described as immunoreactive for the extracted form of ECP in activated eosinophils, our studies have shown that it is also immunoreactive with the granular stored form of ECP, similarly to a recent study by Nakajima *et al* (25). In addition, we chose to investigate the protein expression for IL-6 and RANTES as representatives of eosinophil-derived cytokines and chemokines, respectively. IL-6 (26,27) and RANTES (28,29) are thought to be important in the development and maintenance of the inflammatory response in the airways.

Our observations suggest that MBP, ECP, IL-6 and RANTES are not expressed at detectable levels in freshly purified CD34$^+$ cells. However, in our hands, they appeared to be fully expressed at the protein level in maturing cells at day 16 of culture. At this stage, all eosinophil-derived mediators included in this study appeared to be associated with large, hollow-cored granules, an observation in agreement with earlier study by Scott *et al.* (22) and Popken-Harris *et al.* (15). Interestingly, on day 16, only a few granules showed exclusive immunostaining for MBP. This may be due to differential mediator storage pattern among immature granules. At later stages of maturation (day 19), granules appeared more condensed with intensely co-localizing immunoreactivity for MBP and all other mediators studied.

It is tempting to speculate that the large granules present at day 19 developing eosinophils may have formed by condensation of numerous hollow-cored granules observed at day 16. Interestingly, at days 19 and 23 of culture ECP showed a slightly distinct pattern of immunostaining from that of MBP, IL-6 and RANTES. Yet at more advanced stages of maturation (day 28 of culture), the distribution of immunoreactivity for ECP largely resembled those of IL-6 and RANTES. This may well indicate differential mediator packaging throughout eosinophil maturation under these culture conditions.

A large proportion of immunoreactivity to these mediators in early stages of culture appeared to localize to cytoplasmic regions in these cells. It is important to emphasize that these mediators may also be stored in small secretory vesicles, which may result in an immunostaining pattern resembling that of "cytoplasmic" distribution. This has been demonstrated for RANTES immunoreactivity, which appeared to localize to a population of small secretory vesicles in mature peripheral blood eosinophils (17).

Although the morphological and biosynthetic properties of maturing human eosinophils have been partially investigated by a number of laboratories (19-22,30,31), our work is the first to examine the differential expression and storage of cytokines/chemokines during eosinophil development. The early expression of these mediators is potentially important, since it may indicate that maturing eosinophils have the potential to exhibit local immuno-effector and immuno-regulatory function during differentiation and maturation in the bone marrow as well as local tissues. Indeed, maturing eosinophils generated from bone marrow-derived CD34$^+$ cells are capable of mediator release at early stages of their development (32). It will be of interest to quantify these mediators, although in the present study this could not be pursued due to practical considerations, such as limited cell numbers and the high viscosity of the methylcellulose used to grow these cells. However, we emphasize that, in this respect at least, cord blood-derived eosinophils *in vitro* are likely to be phenotypically distinct from those produced within the bone marrow *in vivo*. The findings of this study, should contribute to our understanding of cytokine synthesis in early progenitor cells as well as developing eosinophils.

References

1. Gleich, G.J, C.R. Adolphson, and K.M. Leiferman. 1993. The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44, 85-101.
2. Wardlaw, A.J., R. Moqbel, A.B. Kay.1995. Eosinophils: biology and role in disease. *Adv. Immunol.* 60, 151-266.
3. Weller, P. 1991. The immunobiology of eosinophils. *N. Engl. J. Med.* 324, 1110-1118.
4. Flavahan, N., N. Slifman, G. Gleich, and P. Vanhoutte. 1998. Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscles: role of the epithelium. *Am. Rev. Respir. Dis.* 138, 685-688.
5. Nakahata, T., A. Gross, and M. Ogawa. 1982. A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. *J. Cell. Physiol.* 113, 455-458.
6. Nakahata, T., and M. Ogawa. 1982. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential colonies. *Proc. Natl. Acad. Sci. USA* 79, 3843-3837.
7. Shalit, M. 1997. Growth and differentiation of eosinophils from human peripheral blood CD34$^+$ cells. *Allerg. Immunol.* 29, 7-10.
8. Shalit, M., S. Sekhsaria, and H.L. Malech. 1995. Modulation of growth and differentiation of eosinophils from human peripheral blood CD34$^+$ cells by IL-5 and other growth factors. *Cell. Immunol.* 160, 50-57.
9. Moqbel, R., F. Levi-Schaffer, and A.B. Kay. 1994. Cytokine generation by eosinophils. *J. Allergy. Clin. Immunol.* 94,1183-1188.

10. Lacy, P., and R. Moqbel. 2000. Eosinophil cytokines. *Chem. Immunol.* 76, 134-155.

11. Ackerman, S.J., D.A. Loegering, P. Venge, I. Olsson, J.B. Harley, A.S. Fauci, and G.J. Gleich. 1983. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* 131, 2977-2982.

12. Lewis, D., J. Lewis, D. Loegering, and G. Gleich. 1978. Localization of guinea pig eosinophil major basic protein to the core of the granule. *J. Cell. Biol.* 77, 702-713.

13. Levi-Schaffer, F., P. Lacy, N.J. Severs, T.M. Newman, J. North, B. Gomperts, A.B. Kay, and R. Moqbel. 1995. Association of granulocyte-macrophage colony-stimulating factor with the crystalloid granules of human eosinophils. *Blood.* 85, 2579-2586.

14. Levi-Schaffer, F., J. Barkans, T.M. Newman, S. Ying, M. Wakelin, R. Hohenstein, V. Barak, P. Lacy, A.B. Kay, and R. Moqbel. 1996. Identification of interleukin-2 in human peripheral blood eosinophils. *Immunology.* 87, 155-161.

15. Dubucquoi, S., P. Desreumaux, A. Janin, O. Klein, M. Goldman, J. Tavernier, A. Capron, and M. Capron. 1994. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J. Exp. Med.* 179, 703-708.

16. Lacy, P., F. Levi-Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J. Velazquez, A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon γ. *Blood.* 91, 2508-2516.

17. Lacy, P., S. Mahmudi-Azer, B. Bablitz, S. Hagen, J. Velazquez, P.F. Man, R. Moqbel. 1999. Rapid mobilization and release of intracellularly stored RANTES in response to interferon-γ in human eosinophils. *Blood.* 94, 23-32.

18. Beil, W.J., P.F. Weller, D.M. Tizizik, S.J. Galli, and A.M. Dvorak. 1993. Ultrastructural immunogold localization of tumor necrosis factor-α to the matrix compartment of eosinophil secondary granules in patients with idiopathic hypereosinophilic synderome. *J. Histochem. Cytochem.* 41, 1611-1615.

19. Saito, H., H. Kiyohiko, H.M. Dvorak, K.M. Leiferman, A.D. Donnenberg, N. Arai, and T. Ishizaka. 1988. Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc. Natl. Acad. Sci. USA* 85, 2288-2292.

20. Dvorak, A., H. Saito, P. Estrella, S. Kissell, N. Arai, and T. Ishizaka. 1989. Ultrastructure of eosinophils and basophils stimulated to develop in human cord blood mononuclear cell cultures containing recombinant human interleukin-5 or interleukin-3. *Lab. Invest.* 61, 116-132.

21. Scott, R.E., and R.G. Horn. 1970. Fine structural features of eosinophilic granulocyte development in human bone marrow. *J. Ultrastruct. Res.* 33, 16-28.

22. Popken-Harris, P., J. Checkel, D. Loegering, B. Madden, M. Springett, G. Kephart, and G.J. Gleich. 1998. Regulation and processing of a precursor form of eosinophil granule major basic protein (ProMBP) in differentiating eosinophils. *Blood.* 92, 623-631.

23. Velazquez, J.R., P. Lacy, S. Mahmudi-Azer, B. Bablitz, C. Milne, and R. Moqbel. 2000. Interleukin-4 and RANTES expression in maturing eosinophils derived from human cord blood CD34+ progenitors. *Immunology.* 101, 419-425.

24. Tai, P., C. Spry, C. Peterson, P. Venge, and I. Olsson. 1984. Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein. *Nature.* 309, 182-184.

25. Nakajima, H., D.A. Loegering, H. Kita, G.M. Kephart, and G.J. Gleich. 1999. Reactivity of monoclonal antibodies EG1 and EG2 with eosinophils and their granule proteins. *J. Leuk. Biol.* 66, 447-454.

26. Yokoyama, A., N. Kohno, S. Fujino, H. Hamada, Y. Inoue, S. Fujioka, S. Ishida, and K. Hiwada. 1995. Circulating interleukin-6 levels in patients with bronchial asthma. *Am. J. Respir. Crit. Care. Med.* 151, 1354-1358.

27. Deetz, D.C., P.J. Jagielo, T.J. Quinn, P.S. Thorne, S.A. Bleuer, and D.A. Schwartz. 1997. The kinetics of grain dust-induced inflammation of the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 155, 254-259.

28. Baggiolini, M., and C.A. Dahinden. 1994. CC chemokines in allergic inflammation. *Immunol. Today.* 15, 127-133.

29. Schall, T.J., K. Bacon, K.J. Toy, and D.V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature.* 347, 669-671.

30. Weller, P., and A. Dvorak. 1994. Human eosinophils: Development, maturation and functional morphology. *In* Mechanisms in asthma and rhinitis. Implications for

diagnosis and treatment. W. Busse, S. Holgate eds. Blackwell Scientific Publication. Inc. Cambridge. MA. 255-275.

31. Dvorak, A., T. Ishizaka, P. Weller, and S. Ackerman. 1993. Ultrastructural contribution to the understanding of the cell biology of human eosinophils: Mechanisms of growth factor-induced development, secretion, and resolution of released constituents from the microenvironment. *In* Eosinophils: Biological and clinical aspects. S. Makino, T. Fukuka eds. CRC Press. Boca Raton, FL.13-22.
32. Scepek, S., and M. Lindau. 1997. Exocytotic competence and intergranular fusion in cord-blood-derived eosinophils during differentiation. *Blood.* 89, 510-518.

Chapter 3

Rapid mobilization of intracellularly stored RANTES in response to interferon-γ in human eosinophils[1]

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[1] Authors, P. Lacy, S. Mahmudi-Azer, B. Bablitz , S.C. Hagen, J. Velazquez, S.F. Paul Man, and R. Moqbel. A version of this chapter was published in *Blood* 1999 94:23-32. I contributed to this work by carrying out a number of the experimental procedures described, including preparation of eosinophils, cell activation, cytospin preparation, immunofluorescent staining and confocal laser scanning microscopy, as well as data analysis. I also helped in the preparation of the final version of the manscript.

Introduction

Eosinophil accumulation is a hallmark of allergic inflammation, particularly within the airway mucosa of asthmatic subjects. Eosinophils are thought to be activated in response to local inflammatory stimuli by releasing an array of mediators. These consist of cytotoxic granule proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO), products of respiratory burst, and lipid mediators (1,2). In addition, eosinophils potentially synthesize or produce up to 18 different cytokines and growth factors, including IL-2 (3,4), IL-4 (5), IL-6 (6,7), GM-CSF (8-10), and RANTES (11-13). A number of these proteins have been shown to exert autocrine effects on eosinophils, including RANTES (8,11,14).

RANTES is a CC chemokine which has been shown to be a potent chemoattractant for $CD4^+/CD45RO^+$ T cells, eosinophils, basophils, monocyte/macrophages, and mast cells (15-18). In addition to eosinophils, RANTES is synthesized and/or released by a number of other cell types, such as T cells, platelets, macrophages, endothelial cells, fibroblasts, epithelial cells, and a mast cell line (16,19-23). Cutaneous injection of RANTES was found to induce marked eosinophil recruitment in human subjects, which was more rapid in allergic compared with normal subjects (24). In earlier studies, RANTES has been implicated in delayed-type hypersensitivity reactions (19), and in ongoing inflammatory processes in rheumatoid arthritis (20). RANTES may have a role in contributing to the infiltration of inflammatory cells in allergen-challenged airway mucosal tissue in asthma. Although the expression and release of RANTES in tissue and bronchoalveolar lavage (BAL) fluids from resting asthmatic and normal subjects do not differ significantly (25,26), the levels of RANTES were found to be elevated in BAL fluids obtained from asthmatics 4 h after allergen challenge (27) and in nasal fluids obtained from subjects with allergic rhinitis following challenge with a grass pollen extract (28). Increased RANTES secretion correlated strongly with elevated tissue eosinophil numbers in both asthma and rhinitis. Furthermore, RANTES has been shown to upregulate expression of CD11/CD18 on monocytes (29) and induce histamine release from human basophils (30), suggesting that it may also have a role in immediate-type allergic responses.

It has previously been shown that peripheral blood eosinophils express mRNA for and release bioactive RANTES in response to serum-coated beads, using immunocytochemistry (ICC), *in situ* hybridization (ISH), and ELISA (11). A granular pattern of immunocytochemical staining of eosinophils was observed, suggesting that eosinophils store preformed RANTES in intracellular compartments. Interferon-γ (IFNγ) was found to upregulate RANTES mRNA and protein expression in eosinophils following 16 h of stimulation (11). IFNγ has been shown to be a viable stimulus for eosinophils in a number of studies (6,7,9,31-33).

We originally hypothesized that intracellular RANTES was secreted following stimulation by IFNγ in a time-dependent manner. However, preliminary data indicated that IFNγ had a more rapid effect on mobilization of RANTES than previously anticipated. Thus, we propose that RANTES is rapidly mobilized from intracellular stores in a piecemeal pattern of degranulation. Eosinophils were purified from peripheral blood obtained from atopic asthmatics and stimulated with recombinant human IFNγ. The release of RANTES was analyzed in cells using a combination of *in vitro* assay, RANTES-specific ELISA, immunogold analysis, confocal laser scanning microscopy (CLSM), and subcellular fractionation, and its localization was compared with that of known crystalloid granule proteins MBP and EPO. Our findings suggest that RANTES, a stored product of eosinophils, is readily and selectively released in a pattern akin to piecemeal degranulation after stimulation.

Materials and Methods

Materials

Mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP) detection kits were obtained from Dako (Glostrup, Denmark). Adenosine triphosphate, aprotinin, Nα-*p*-tosyl-L-arginine methyl ester (TAME), phenylmethylsulfonyl fluoride (PMSF), Fast Red TR, leupeptin, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, β-nicotinamide adenine dinucleotide (reduced form), sodium pyruvate, and tetramethylbenzidine (TMB) substrate solution were purchased from Sigma (Oakville, ON, Canada). Genistein was obtained from Calbiochem Corporation (San Diego, CA, USA). Recombinant human IFNα was a kind gift from Dr. Aziz Ghahary, Department of Surgery, University of Alberta. Nycodenz was purchased from Gibco BRL Life Technologies, Ltd (Grand Island, NY, USA). All reagents used in this study, including media, were negative for LPS activity, as determined using the E-Toxate assay (Sigma).

Preparation of eosinophils

Peripheral blood (100 ml) was obtained from mild atopic asthmatic and atopic nonasthmatic subjects displaying eosinophilia >5%, and who were not receiving oral corticosteroids. Following red blood cell sedimentation in 5% dextran, remaining cells were subjected to density centrifugation on Ficoll. Eosinophils were then purified from the granulocyte pellet by immunomagnetic selection using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Highly purified CD16$^-$ eosinophils (>99%) were obtained by negative selection, depleted of neutrophils using anti-CD16-conjugated immunomagnetic beads as described previously (7,10,34). Contamination by mononuclear cells and lymphocytes was avoided by co-incubation with anti-CD14 and anti-CD3-coated micromagnetic beads (Miltenyi Biotec).

In vitro assay of granule protein release from eosinophils

Purified human eosinophils were aliquoted at 2 x 10^6 cells/tube and treated with 500 U/ml IFNγ at 37°C for various times in RPMI-1640 (BioWhittaker, Walkersville, MD, USA). The reaction was terminated by placing tubes on ice and centrifuging cells at 400*g* for 5 min at 4°C. Assays were then carried out for RANTES immunoreactivity in supernatants using a Quantikine ELISA kit (R & D Systems, Minneapolis, MN, USA) with a detection sensitivity of 31.2 pg/ml. For detection of two other crystalloid granule proteins, EPO and β-hexosaminidase, assays for these were a modification of those carried out previously (4,7,10,35). Briefly, EPO activity was assayed using TMB substrate solution by combining 50 μL sample with 150 μL substrate solution in a 96-well microplate and incubating at room temperature for 15 min. The reaction was terminated by 50 μL 4 M sulfuric acid and absorbance was read at 450 nm in a spectrophotometric microplate reader (Vmax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). For β-hexosaminidase activity, present in both secretory and lysosomal granules, 50 µl sample was added to each well of a 96-well microplate and mixed with 50 µl substrate solution (1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide in 0.2 M citrate buffer, pH 4.5, and 0.1% Triton X-100) before incubating (37°C, 1-2 h). The reaction was terminated by the addition of 150 µl ice-cold 0.2 M Tris, and the fluorescence (excitation 360 nm, emission 460 nm) measured in a Millipore CytoFluor 2350 plate reader (Millipore, Nepean, ON, Canada).

Immunogold labeling and electron microscopy

This procedure was a modification of a previously described technique (5). Briefly, pelleted isolated eosinophils were fixed in freshly prepared formaldehyde (2% in phosphate buffer, 0.1 M, pH 7.4 [PB]) for 2 h, embedded in Lowicryl K4M resin, and subjected to an infiltration procedure involving a progressive lowering of the temperature. Silver sections were cut and picked up onto Formvar-coated copper grids. Before labeling, sections were blocked for 10 min with 0.14% glycine in PB. Additional blocking was done for 10 min in 3% horse serum. Grids were then floated on a solution containing 20 µg/ml mouse monoclonal anti-human RANTES antibody (R & D Systems) for 2 h. A further blocking step was performed using 0.14% glycine in PB. The immunoreactive label was visualized by goat anti-mouse antibody conjugated to gold particles (20 nm diameter; E-Y Laboratories, Inc., San Mateo, CA, USA) at 0.5 µg/ml in PB for 2 h. Sections were washed 3 times with 0.14% glycine in PB before being rinsed for 3 min with distilled water. For negative controls, we substituted anti-RANTES with mouse IgG1 as the isotype control (20 µg/ml; R & D Systems). Staining with osmium tetroxide was omitted to ensure visualization of gold particles in electron-dense crystalloid granules in eosinophil sections.

Double labeling and confocal laser scanning microscopy

Cytospins of eosinophils (100 μl of 0.5 x 10^6 cells/ml in RPMI supplemented with 20% FCS) were made by spinning slides in a Cytospin 2 (Shandon Ltd, Astmoor, Runcorn, UK) centrifuge (800 rpm for 2 min) followed by fixing in 2% paraformaldehyde in PBS for 6 min. These fixing and staining procedures were optimized as previously reported (36), and are satisfactory for visualization of granule proteins in the absence of a permeabilization step. In our hands, permeabilization agents were detrimental in obtaining optimal cell morphology. Slides were then subjected to a wash step (five washes in Tris-buffered saline [TBS], pH 7.4), followed by incubation in blocking solution (2% BSA) in a humidified container for 1 h. Specific antibody binding was carried out for 1 h with TBS containing 5 μg/ml mouse monoclonal anti-human RANTES antibody (R & D Systems). Immunoreactivity to RANTES was detected using 20 μg/ml BODIPY FL-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA) as previously optimized by our laboratory (36). Slides were blocked again for 2 h using 50 μg/ml goat anti-mouse IgG (Molecular Probes) and double-labeled with 1% mouse monoclonal anti-human MBP antibody (BMK-13) in TBS for 1 h. Bound BMK-13 was detected by incubating 5 μg/ml Texas Red-labeled goat anti-mouse antibody for 1 h (Caltag Laboratories, San Francisco, CA, USA). Mouse IgG1 (5 μg/ml) was used as an isotype control (R & D Systems). After a final wash step, 10 μl of antibleaching agent (0.4% *n*-propyl gallate [Sigma] in 3:1 glycerol:TBS) was applied to each slide before coverslip attachment. Slides were examined using a 100X objective under a Leica confocal laser scanning microscope (CLSM; Heidelberg, Germany). Images were collected and processed as previously described (7).

Subcellular fractionation

Purified peripheral blood eosinophils were homogenized by repeated passages through a ball-bearing cell homogenizer, with resulting organelles separated by linear density gradient as described in earlier reports (4,5,7,10). Briefly, at least 5 x 10^7 purified eosinophils from asthmatics were suspended in ice-cold 0.25 M HEPES-buffered sucrose (containing 10 mM HEPES, 1 mM EGTA, pH 7.4) and centrifuged at 4°C. Cells were resuspended in homogenization buffer (HEPES-buffered sucrose described above, supplemented with 100 µg/ml PMSF, and 5 µg/ml each of leupeptin, aprotinin, and TAME, 2 mM $MgCl_2$ and 1 mM ATP) to 10-15 x 10^6/ml, and subjected to 10-20 passes through a 12 µm clearance in a ball bearing homogenizer (EMBL, Heidelberg, Germany). The homogenate was centrifuged at 400 *g* for 10 min, and the resulting postnuclear supernatant was layered onto an 8-ml linear Nycodenz gradient (0-45% Nycodenz dissolved in HEPES-buffered sucrose with protease inhibitor cocktail) in a Beckman 14 x 89 mm Ultra-Clear™ centrifuge tube (Beckman, Palo Alto, CA, USA). The gradient was subjected to equilibrium density centrifugation at 100,000 *g* for 1 h at 4°C, and 24 x 0.4 ml fractions were collected from each preparation and stored at -80°C until used.

Marker enzyme assays

Three marker enzyme assays were used to obtain profiles of specific subcellular organelles in fractions collected from density gradient centrifugation. Activities of EPO and β-hexosaminidase were determined in supernatants and pellets using the same technique described earlier in this section. Cytosolic activity was determined using a modification of a microtitre plate endpoint assay (37) for lactate dehydrogenase (LDH), where 10 μL of sample was mixed with 80 μL 1 mg/ml NADH and 0.75 mM pyruvate in pH 7.5 phosphate buffer in a microtitre plate and incubated at 37°C for 30 min, followed by the addition of 80 μL 0.2 mg/ml 2,4-dinitrophenylhydrazine in 1 M HCl and incubation at room temperature for 20 min. The reaction was terminated by the addition of 40 μL 3.5 M NaOH and absorbance read at 450 nm. Plasma membrane activity was determined by dot blot analysis (see below) with monoclonal antibody to CD9 as previously described (4,5,7,10).

Dot blot analysis

Dot blot analysis was carried out to determine the distribution of CD9 in subcellular fractions of resting and stimulated eosinophils. Anti-CD9 mAb (purified IgG2a) was kindly provided by Dr. A.R.E. Shaw (Cross Cancer Institute, University of Alberta). A mouse monoclonal IgG2a isotype control was used as the negative control for anti-CD9 (Pharmingen Canada, Mississauga, ON, Canada). Samples of each fraction (2 μL) were pipetted onto nitrocellulose strips, allowed to dry and blocked in 5% milk powder. Blocked membrane strips were incubated with 1:1000 anti-CD9 antibody, and after extensive washings in TBS, pH 7.6 + 0.2% Tween-20, were developed using APAAP staining technique as previously described (10). The fractional activities of anti-CD9 immunoreactivity were assessed by staining density using a gel scanner, given arbitrary units and converted to percentage of total activity in all fractions.

Data presentation

The bioactivity of eosinophil granule, membrane and cytosol constituents following fractionation are expressed as frequency distributions as previously described (10). RANTES was quantitatively displayed as pg/fraction and pg/ml in fractions and supernatants, respectively. Statistical comparisons were carried out using the Mann-Whitney test (one-tailed) and the Kruskal-Wallis one-way analysis of variance. Results were considered significant when $p < 0.05$.

Results

Immunocytochemistry using APAAP

In cytospin preparations of antibody-specific staining of buffy coat from asthmatic subjects, RANTES immunoreactivity was detected in eosinophils using APAAP staining. Morphologically identifiable eosinophils, but not neutrophils or lymphocytes, displayed a mixture of granular and extragranular staining corresponding to RANTES immunoreactivity (Fig. 1A). This indicates that while RANTES appears to be stored in association with the unique crystalloid granules of eosinophils, it may also be found in a number of other intracellular compartments. The isotype control antibody (mouse IgG1) was negative (Fig. 1B).

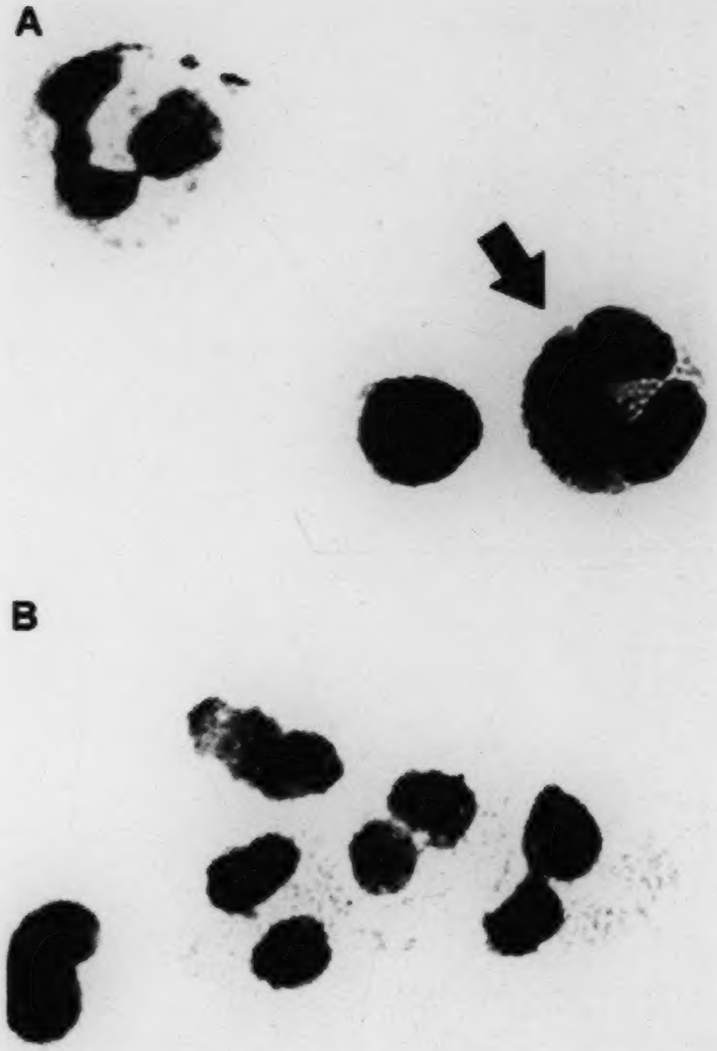

Fig 1.3. Human eosinophil detected in a buffy coat cytospin. (A) The eosinophil was stained specifically with mouse monoclonal anti-human RANTES (20 µg/mL) using APAAP staining, as indicated by the arrow. Two unlabeled cells are also visible within this field, a neutrophil possessing a multilobed nucleus (upper left) and a lymphocyte (left of the stained eosinophil). (B) Isotype control using mouse IgG1 antibody (20 µg/mL). Original magnification × 100.

Release of RANTES following IFNγ stimulation, in vitro

Incubation of eosinophils in culture media containing human recombinant IFNγ for prolonged periods (up to 24 h) has been previously demonstrated to upregulate the expression of a number of eosinophil-derived cytokines, including RANTES (11). In order to evaluate the time course of RANTES release during short periods of stimulation by IFNγ (500 U/ml), the amount of RANTES released by stimulated cells was measured at 0, 10, 30, 60, 120, and 240 min (2 X 10^6 cells/time point). IFNγ induced a rapid release of RANTES from human eosinophils, reaching maximal levels after 60-120 min (4 experiments). In a representative experiment, eosinophils released an average of 80 ± 15 pg/ml RANTES following 120 min of IFNγ stimulation ($p < 0.05$; Fig. 2A). The amount of maximal release varied between the 4 donors (range = 74.5-302 pg/ml). After 2 h stimulation, the amount of RANTES detected in supernatants was diminished to baseline values. Unstimulated eosinophils showed no significant spontaneous release of RANTES (Fig. 2A). Thus, IFNγ was found to induce rapid *in vitro* release of RANTES from human peripheral blood eosinophils. In comparison, the release of EPO was assayed in the same supernatants and plotted as a percentage of release induced by 60 min of incubation at 37°C with a maximally-stimulating agonist, serum-coated Sephadex beads, in a separate sample. EPO release was potently induced by IFNγ within 60 min of incubation and diminished after 120 and 240 min of incubation. However, since EPO activity is not always stable following its release (38,39), we also measured β-hexosaminidase activity in these supernatants. β-hexosaminidase activity continued to increase in supernatants during continuous incubation, reaching values exceeding the levels induced by serum-coated Sephadex beads, after 240 min of stimulation by IFNγ (Fig. 2B).

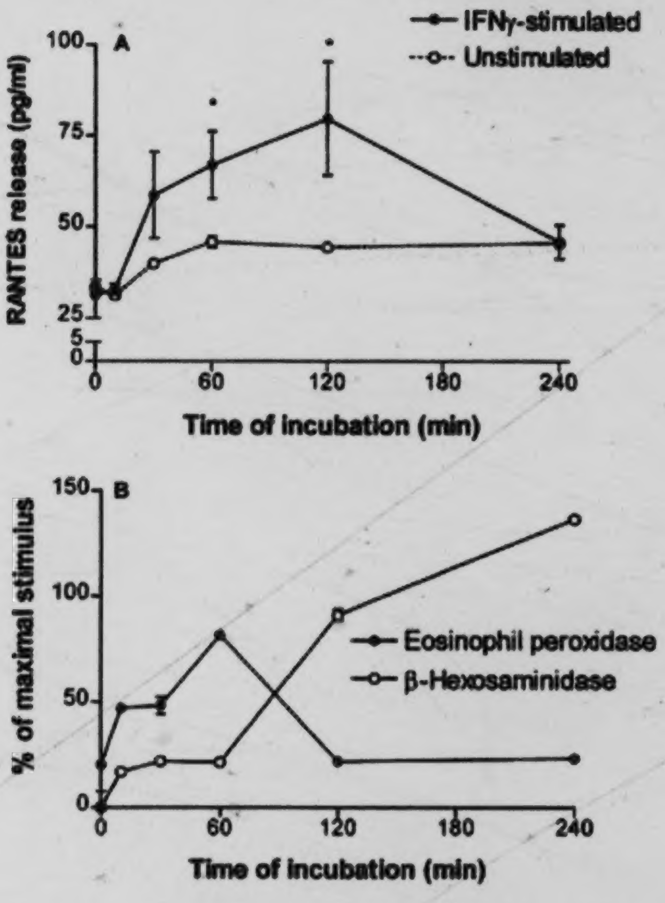

Fig 2.3. Time course of RANTES, EPO, and β-hexosaminidase release from human peripheral blood eosinophils induced by 500 U/mL recombinant human IFN-γ. (A) RANTES immunoreactivity in supernatants of stimulated eosinophils (2×10^6 cells/time point) was detected using a specific ELISA. Values represent averages of triplicate measurements from cells at 0, 10, 30, 60, 120, and 240 minutes of incubation obtained from a representative donor. A similar trend of release was observed in four separate donors. The dotted line represents a single measurement of spontaneous release of RANTES from eosinophils (duplicate values given for times 0 and 60 minutes). * $P < .05$ compared with RANTES measured in supernatants at the start of the time course

by Kruskal-Wallis one-way analysis of variance. The detection sensitivity of the RANTES ELISA was 31.2 pg/mL. (B) IFN-γ also induced the release of eosinophil peroxidase and another granule-stored product, β-hexosaminidase, detected in the same IFN-γ-stimulated supernatants shown in (A). The amount of granule protein release is expressed as a percentage of the release induced by a maximally stimulating agonist (serum-coated Sephadex beads) in another sample. Points and error bars represent the mean and standard error of mean (SEM) of at least three measurements.

Immunogold labeling of RANTES

Unstimulated eosinophils exhibited a granular staining pattern for RANTES immunoreactivity, as indicated by the proximity of gold particles to electron-dense granule cores (Fig. 3A, B). This pattern of immunolabeling suggests that RANTES may be stored in association with crystalloid granules. In addition, RANTES-specific staining was distributed throughout the cell in the extragranular milieu (shown by the arrowhead in Fig. 3A). The isotype control showed negligible background (Fig. 3C).

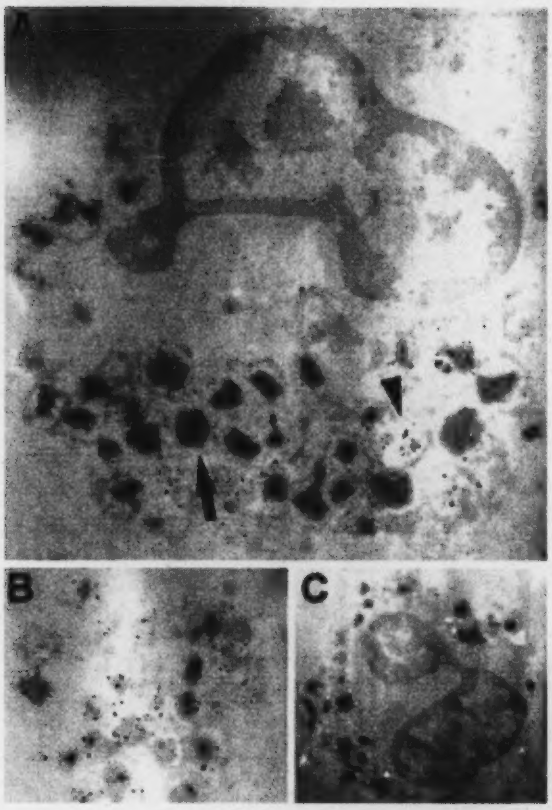

Fig 3.3. Transmission electron microscopy of immunogold-labeling of RANTES in unstimulated eosinophils. (A) The arrow indicates gold particles associated with electron-dense crystalloid granules, while the arrowhead shows immunogold labeling of extragranular areas. (B) Higher magnification of another cell. (C) Isotype control using mouse IgG1 antibody. Original magnifications: (A), ×9,100; (B), ×34,000; and (C), ×6,900.

Confocal laser scanning microscopy (CLSM)

To examine mobilization of stored RANTES using MBP as a marker for eosinophil crystalloid granule, resting and IFNγ-stimulated eosinophils were subjected to immunofluorescent labeling with appropriate antibodies. Immunostained cells displayed discrete green and red labels, which correspond to BODIPY FL-conjugated RANTES (Fig. 4A) and Texas Red-conjugated MBP (Fig. 4B) immunoreactivity, respectively. Where colors overlapped in the combined images, the immunofluorescence appeared yellow (Fig. 4C), suggesting that the two labeled proteins co-localize to the same intracellular compartment. The isotype control exhibited negligible immunoreactivity after subtraction of autofluorescence as previously reported [data not shown; (36)]. At higher magnifications, crystalloid granules appeared doughnut-shaped, with red centers (crystalline core MBP) surrounded by green immunofluorescence to RANTES, corresponding to the core and matrix of the crystalloid granules, respectively (Fig. 4D, E, & F). There was only partial overlap between MBP and RANTES immunoreactivities in the granules.

In time course experiments, eosinophils stimulated with 500 U/ml IFNγ for 0, 5, 10, 30, 60 min and 16 h were fixed and stained for RANTES and MBP immunofluorescence. Interestingly, after 10 min of incubation with IFNγ, RANTES immunofluorescence was redistributed around the periphery of the cells (Fig. 4I, M, & O), while that of MBP remained within the core of the secretory granules. The RANTES-specific immunofluorescence became visibly depleted in cells after 1 h of incubation with IFNγ, although MBP activity was still detectable (Fig. 4K). After 16 h of incubation, RANTES immunoreactivity returned, indicating that some degree of replenishment may have occurred (Fig. 4L). These findings were reproduced in eosinophils from 3 asthmatic donors, and images represent the labeling pattern of the majority of cells in cytospin preparations.

To test the specificity of the IFNγ response, eosinophils were incubated with genistein (10^{-6} M) for 10 min prior to adding 500 U/ml IFNγ for 10 min. Genistein is a broad specificity tyrosine kinase inhibitor used to inhibit early steps in the IFNγ receptor signaling pathway following ligand binding (40). It fully inhibited the effects of IFNγ on RANTES immunoreactivity in single-labeled cells (Fig. 4P; a stimulated single-labeled cell is shown for comparison in Fig. 4O). Inhibition by genistein was detected in > 90% of cells examined by CLSM. In confirmation of this, we have also observed that genistein (10^{-6}M) inhibited RANTES release from eosinophils *in vitro* (2×10^6) by 32% after 120 min of IFNγ incubation (data not shown). In addition, we incubated eosinophils with IFNα (1000 U/ml) for 10 min and found that it had no observable effect on the distribution of RANTES immunoreactivity in eosinophils (Fig. 4Q). We took advantage of the small numbers of cells required for study by CLSM to examine the effects of IFNα and genistein on eosinophils, since other *in vitro* techniques require substantially larger numbers of cells. In a separate assay, recombinant human IL-3 (25 ng/ml), IL-5 (10 ng/ml), and GM-CSF (10 ng/ml) did not induce significant RANTES release from eosinophils after 1 h of stimulation (data not shown).

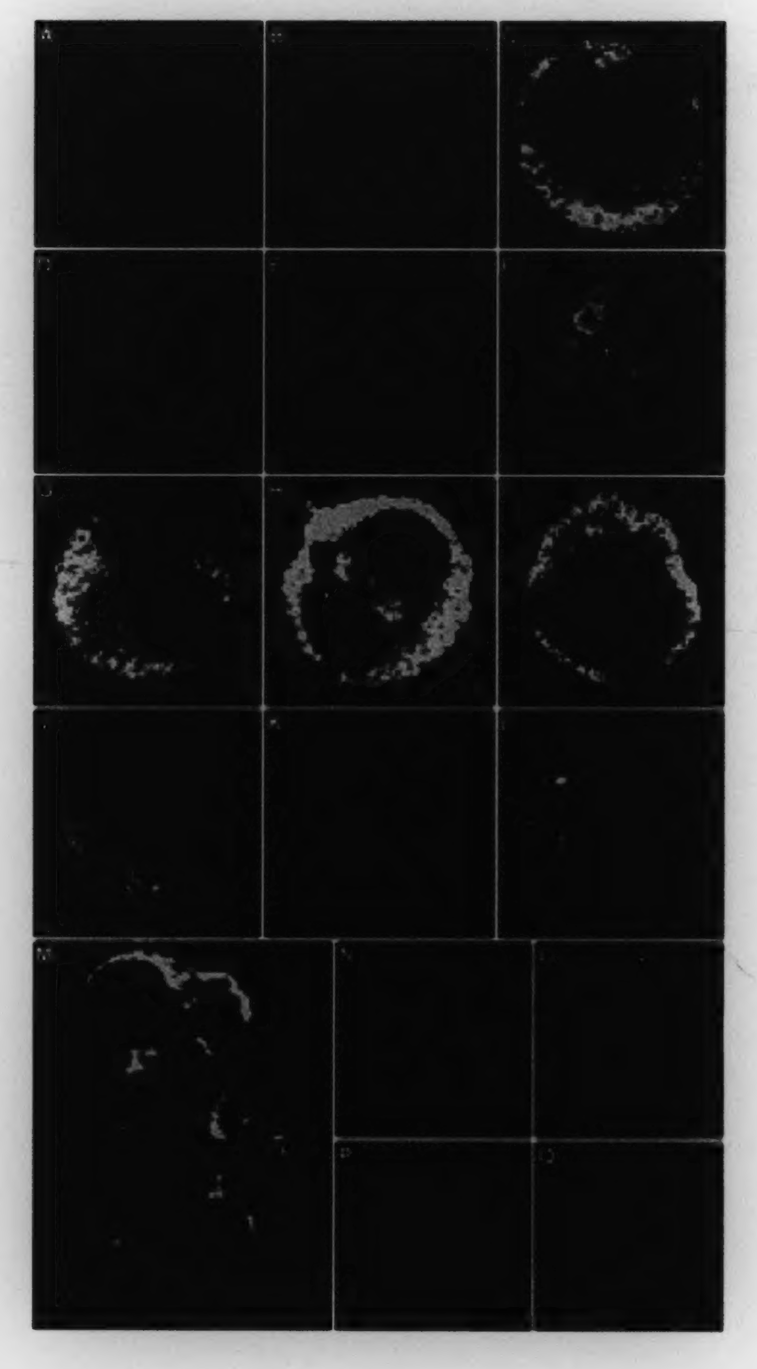

Fig 4.3. CLSM of immunofluorescence staining of eosinophils. (A through C) Unstimulated eosinophils labeled with BODIPY FL indicating RANTES immunoreactivity (A), Texas Red corresponding to MBP (B), and combined images (C). (D through F) Higher magnification of eosinophil crystalloid granules showing matrix-associated doughnut-shaped RANTES immunoreactivity (D), surrounding red-labeled cores of MBP immunoreactivity (E), and combined images of the same structure (F). (G through L) Combined images of RANTES and MBP, depicting time course of IFN-γ (500 U/mL) stimulation, comparing (G) unstimulated cells with those stimulated for 5 minutes (H), 10 minutes (I), 30 minutes (J), 60 minutes (K), and 16 hours (L). (M) Lower magnification of combined images of eosinophils stimulated for 10 minutes with IFN-γ. (N) Single-labeled unstimulated eosinophil, compared with (O) cell after 10 minutes stimulation (500 U/mL IFN-γ). (P) Inhibitory effect of 10^{-6} mol/L genistein added for 10 minutes before IFN-γ stimulation. (Q) Cell incubated with 1,000 U/mL IFN-α for 10 minutes. Original magnification × 100 for all images, except for (M), ×63.

Subcellular fractionation

Eosinophils (5 x 10^7) were homogenized, loaded onto gradients of Nycodenz (0-45%) for ultracentrifugation, and fractions containing intact organelles collected for later analysis by ELISA and assays for enzyme activity. Intracellular compartments were identified in gradients by measuring marker enzyme activities within individual fractions (Fig. 5). Crystalloid secretory granules were measured using assays for EPO and β-hexosaminidase, while plasma membrane activity was determined by dot blot analysis of the fractions using anti-CD9. Cytosolic fractions were detected using an endpoint assay for LDH. Fractions with plasma membrane activity are known to contain other light membranes including Golgi compartments, as determined by galactosyl transferase activity measured in subfractionated guinea pig eosinophils (41). We have previously shown that the pellet produced from pooled fractions corresponding to peak granule protein activity, which sediment at high buoyant densities typically observed for crystalloid granules, is enriched in secretory granules (10).

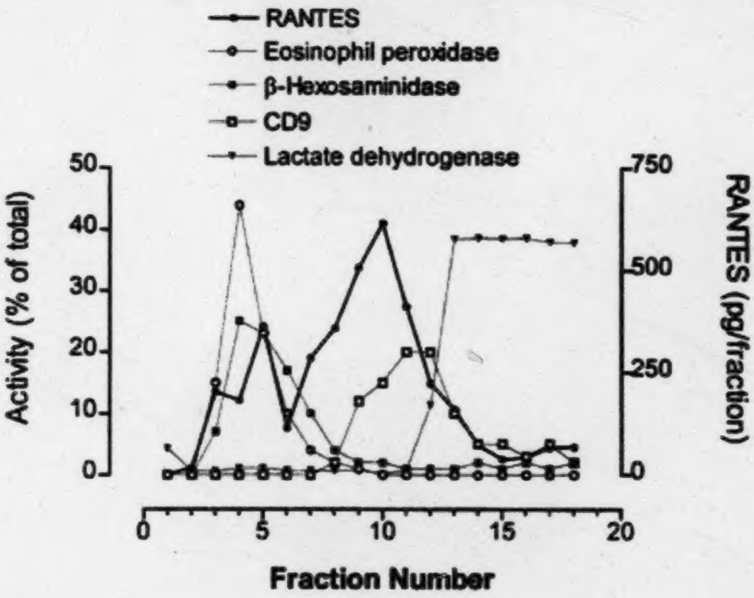

Fig 5.3. Subcellular fractionation of unstimulated peripheral blood eosinophils (5×10^7) obtained from an asthmatic donor. Fractions were collected from a 0% to 45% linear Nycodenz gradient and analyzed for marker enzyme activities to obtain profiles of subcellular compartments. Marker assays used were eosinophil peroxidase (secretory granules), β-hexosaminidase (secretory granules and lysosomal granules), CD9 (plasma membrane), and lactate dehydrogenase (cytosol). Quantification of RANTES was performed by ELISA for each fraction and is expressed as pg/fraction.

In unstimulated eosinophils, RANTES immunoreactivity was detected in at least two separate intracellular compartments (Fig. 5 & 6A). The first peak of RANTES-specific activity was detected in secretory granule-rich fractions, determined by EPO and β-hexosaminidase activity, while a larger peak was found to be associated with the light membrane fractions, which overlapped with CD9 immunoreactivity. Some CD9 immunoreactivity was visible in the granule fractions, as described earlier (4,10,42), although its optical density was too low to be detected, suggesting that a small amount of CD9 is also intracellularly distributed in the eosinophil. This observation confirmed our results from immunogold labeling and CLSM, which suggested that RANTES immunoreactivity only partially co-localized with secretory granules in unstimulated cells. Unstimulated eosinophils in this example were found to store approximately 72 pg RANTES/10^6 cells.

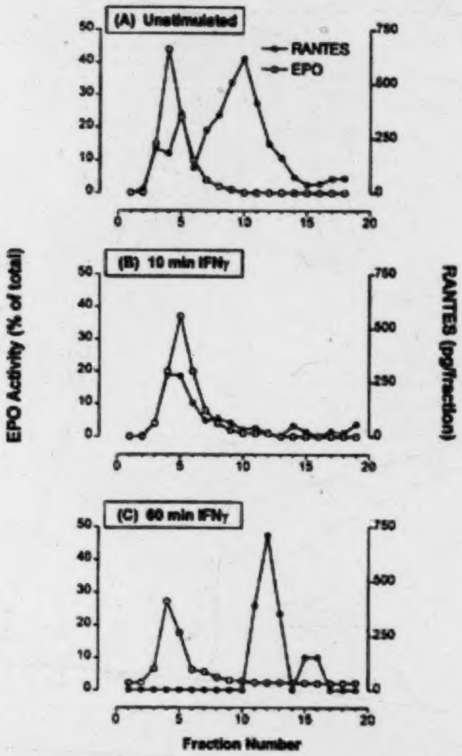

Fig 6.3. Subcellular fractionation of resting and IFN-γ-stimulated eosinophils (5×10^7 per fractionation). These experiments were conducted at different times using purified blood eosinophils from the same donor. Immunoreactivity to RANTES was determined in individual fractions by ELISA and expressed as pg/fraction. Profiles of EPO activity are shown here for comparison. (A) Unstimulated eosinophils, followed by eosinophils stimulated for (B) 10 minutes and (C) 60 minutes with 500 U/mL IFN-γ. Stimulation with IFNγ (500 U/ml) induced a striking change in the distribution of RANTES immunoreactivity in subfractionated eosinophils. RANTES was rapidly depleted from light membrane-associated fractions following 10 min of IFNγ stimulation (Fig. 6B), while some immunoreactivity remained within the granule-associated fractions. Eosinophils stored approximately 23 pg/10^6 cells of RANTES-specific activity following

IFNγ stimulation for 10 min, a reduction of 68% compared with unstimulated cells. Moreover, eosinophils stimulated for 60 min with IFNγ showed a marked loss of RANTES immunoreactivity from fractions containing peak secretory granule activity (Fig. 6C), which was translocated to plasma membrane-associated fractions at 49% of the quantity measured in unstimulated cells (35 pg RANTES/10^6 cells). These results are in agreement with those of CLSM, in which eosinophils displayed reduced RANTES activity after stimulation with IFNγ (Fig. 4J, K). Each subfractionation profile was prepared on different occasions from the same donor to allow comparison of control and stimulated cells. IFNγ-stimulated eosinophils showed similar profiles of EPO activity to that of unstimulated cells (Fig. 6), although the peak of EPO activity appeared to be partially diminished after 60 min of IFNγ stimulation.

Discussion

We have shown for the first time that RANTES immunoreactivity in human eosinophils is associated with the matrix of the crystalloid granules. This is based on its close apposition to the crystalline core granule protein marker, MBP. Interestingly, RANTES was also detected in an extragranular compartment distinct from MBP- and EPO-containing granules, which was readily released in response to IFNγ. We propose that the rapidly mobilizable RANTES is contained within a putative pool of small secretory vesicles that is physically distinct from crystalloid granule.

The profile of RANTES immunoreactivity in fractions from unstimulated eosinophils suggests that the larger peak is likely to be associated with small, light-density vesicles, which possess a greater buoyant density than that of the plasma membrane, as indicated by CD9 immunoreactivity. It is important to note that these fractions do not fully discriminate between endosomal membranes, Golgi, and plasma membrane (41,43). The light membrane fractions are also likely to contain the vesiculotubular structures previously described in eosinophils (44).

IFNγ was observed to activate the release of RANTES from eosinophils in parallel with two other granule-associated proteins, EPO and β-hexosaminidase. Invariably, the levels of RANTES in these supernatants diminished to baseline values after 240 min of incubation. Those of EPO were similarly found to become reduced after 120 min of incubation (Fig. 2). These observations suggest that both RANTES and EPO may be sequestered by surfaces within the assay after their release. Released EPO is likely to be lost to surfaces due to its inherent highly cationic nature (pI of 10.8) (38,39). In addition, RANTES is rapidly sequestered by cell surface glycosaminoglycans following its secretion, which then foster the adhesion and activation of RANTES-responsive cells (45). In our subfractionation studies, immunoreactivity to RANTES was observed to shift to a very low density peak after 60 min of stimulation with IFNγ, which was shifted to the right in comparison with the light density peak of RANTES in unstimulated cells (Fig. 6A & C). This shift in the density of RANTES immunoreactivity suggests that released RANTES may be adhering to the glycosaminoglycans coating the surfaces of eosinophils. In support of the possibility that specific eosinophil products may be lost to surfaces during *in vitro* assay, the non-cationic granule-derived enzyme β-hexosaminidase (predicted pI of 5.4-5.9) was found to increase in supernatants during incubation with IFNγ (Fig. 2B).

Evidence for the existence of a putative small secretory vesicle in eosinophils was provided in our studies on the effects of IFNγ on RANTES mobilization by CLSM. RANTES immunoreactivity appeared to be transferred to the periphery of cells during IFNγ stimulation, apparently to a different vesicular compartment from the crystalloid granules. Moreover, the subcellular fractionation profile of RANTES in IFNγ-stimulated cells (after 10 min) showed that much of the RANTES associated with light density fractions was depleted, while granule-associated RANTES was maintained at a level equivalent to that of unstimulated cells. The observation that IFNγ exerted such a rapid effect on mobilization and release of eosinophil-derived RANTES was novel and compelling. This is complementary to an earlier report, in which IFNγ (1000 U/ml) upregulated RANTES mRNA and protein expression within eosinophils after 16 h of stimulation (11), and provides further support for our recent finding that IFNγ rapidly elevated IL-6 immunoreactivity in human peripheral blood eosinophils (7). After 60 min of stimulation by IFNγ, nearly all the detectable RANTES immunoreactivity was co-localized with very light density membranes as determined by subcellular fractionation, indicating that the crystalloid granule-associated RANTES may have been selectively removed and transported via small secretory vesicles.

These observations suggest that eosinophils possess a unique mechanism for selective, piecemeal release of mediators from the crystalloid granules, probably through exocytosis of a population of small, light-density vesicles. Such small secretory vesicles may be responsible for shuttling crystalloid granule proteins from the granules to the plasma membrane. Selective release of eosinophil granule proteins has been described in earlier reports (46). A similar pattern of piecemeal degranulation has been proposed based on electron microscopy sections of eosinophilic degranulation, *in vivo* (44). Previous studies have shown that eosinophils undergo degranulation in response to intracellularly applied agonists, for example, GTPγS (35,47), although the mechanisms regulating exocytotic release have not yet been fully elucidated. We are currently investigating the identity of the putative small secretory vesicles with a view to determining their precise co-localization with known eosinophil-derived intracellular proteins.

IFNγ has been shown to stimulate eosinophils *in vitro*, as shown in its ability to augment eosinophil-induced ADCC (31) and induce the expression of FcγRIII (CD16) (32), and CD69 (33). Further, IFNγ has been demonstrated to stimulate the release and/or upregulation of a number of cytokines from eosinophils, including IL-3 (48), IL-6 (6,7), GM-CSF (8,9), and RANTES (11). Expression of functional IFNγ receptors on human peripheral eosinophils has been described recently (49). These and future experiments continue to contribute to the intriguing observation that IFNγ can induce rapid changes in cytokine expression, receptor upregulation, and mediator release in eosinophils.

Activation of RANTES mobilization and release from eosinophils by IFNγ was specific, as shown by genistein inhibition, a broadly specific tyrosine kinase inhibitor, suggesting that IFNγ acts on these cells via the IFNγ receptor which activates the Jak-STAT pathway (40). Furthermore, other cytokines such as IFNα, IL-3, IL-5, and GM-CSF were found to have little or no effect on RANTES immunoreactivity or release into supernatants (Fig. 4Q; data not shown). The effects of IFNγ were unlikely to be LPS-mediated since the culture media containing IFNγ used in this study, as well as all other media, were negative for LPS as determined by routine E-Toxate testing (data not shown).

All eosinophils tested in these experiments were derived from subjects exhibiting atopy. It is possible that the effects of IFNγ on RANTES mobilization were due to enhanced susceptibility of the cells to IFNγ because of priming, for example (50). However, we have also detected IFNγ-induced release of intracellularly stored RANTES from eosinophils obtained from atopic nonasthmatic subjects. Thus, we conclude that it is unlikely that the capacity of eosinophils to generate a differential response to IFNγ is dependent on the asthmatic status of the donor.

In both human and murine studies, cytokines released during immune and inflammatory reactions have been proposed to follow a dichotomy of Th1 and Th2-type responses depending on the nature of the stimulus delivered to the immune system. Release of IFNγ has been associated with Th1-type cytokine responses in bacterial and viral infections along with the suppression of atopy (51,52). On the other hand, eosinophils are regarded as Th2-type effector cells with the potential to respond to Th2-type cytokines thought to be associated with the allergic phenotype (2,53,54). However, the distinction between Th1 and Th2 cytokine profiles in most cases of infection and inflammation in humans is less clearcut. Thus, while IFNγ potently inhibits granulocytic maturation and proliferation in the bone marrow (55), the levels of IFNγ have been found to be increased in the sera of patients with acute severe asthma, who also exhibit lung and tissue eosinophilia (56,57). The significance of these findings may be that IFNγ has a role in regulation of eosinophil homeostasis by stimulating fully mature eosinophils locally while preventing excessive eosinophilia, as suggested by Valerius *et al.* (31). It is tempting to speculate that IFNγ released from virus-infected inflammatory and immune cells within the airway mucosa in asthmatics may contribute to activation of resident airway eosinophils during viral exacerbation of asthmatic attacks (58).

Eosinophil-derived RANTES is likely to play a role in paracrine, autocrine, or juxtacrine signaling following its release, and its bioactivity on other eosinophils, at least *in vitro*, has previously been demonstrated (11). Besides RANTES, eosinophils contain a number of other cytokines in their secretory granules, such as IL-2, IL-4, IL-5, IL-6, TNFα, and GM-CSF (4,5,7,10,59,60). Storage of cytokines as preformed mediators within secretory granules and their rapid release following stimulation may lend eosinophils the potential to regulate local inflammatory responses. Many of eosinophil-derived cytokines so far described are produced in smaller proportions than those of other immune cells, such as T cells. However, unlike eosinophils, T cells are not known to have the capacity to store cytokines. Cytokines produced from an overwhelming influx of actively degranulating eosinophils into the airways in asthma, for example, are likely to prolong their own survival and perpetuate the inflammatory response. These exciting observations will be important in expanding our knowledge of the cytokine and chemokine network that regulates the processes of eosinophil activation and subsequent secretion of cytokines, chemokines, and especially granule proteins with their recognized damaging sequelae in allergic inflammation.

References

1. Gleich, G.J., C.R. Adolphson, and K.M. Leiferman. 1993. The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44, 85-101.
2. Wardlaw, A.J., R. Moqbel, and A.B. Kay. 1995. Eosinophils: biology and role in disease. *Adv. Immunol.* 60, 151-266.
3. Bossé, M., M. Audette, C. Ferland, G. Pelletier, H.W. Chu, A. Dakhama, S. Lavigne, L.P. Boulet, and M. Laviolette. 1996. Gene expression of interleukin-2 in purified human peripheral blood eosinophils. *Immunology.* 87,149-154.
4. Levi-Schaffer, F., J. Barkans, T.M. Newman, S. Ying, M. Wakelin, R. Hohenstein, V. Barak, P. Lacy, A.B. Kay, and R. Moqbel. 1996. Identification of interleukin-2 in human peripheral blood eosinophils. *Immunology.* 87, 155-161.
5. Moqbel, R., S. Ying, J. Barkans, T.M. Newman, P. Kimmitt, M. Wakelin, L. Taborda-Barata, Q. Meng, C.J. Corrigan, S.R. Durham, and A.B. Kay. 1995. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *J. Immunol.* 155, 4939-4947.
6. Hamid, Q., J. Barkans, Q. Meng, S. Ying, J.S. Abrams, A.B. Kay, and R. Moqbel. 1992. Human eosinophils synthesize and secrete interleukin-6, *in vitro*. *Blood.* 80,1496-1501.
7. Lacy, P., F. Levi-Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J. Velazquez, A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon γ. *Blood.* 91, 2508-2516.

8. Kita, H., T. Ohnishi, Y. Okubo, D. Weiler, J.S. Abrams, and G.J. Gleich. 1991. Granulocyte/macrophage colony-stimulating factor and interleukin 3 release from human peripheral blood eosinophils and neutrophils. *J. Exp. Med.* 174, 745-760.

9. Moqbel, R., Q. Hamid, S. Ying, J. Barkans, A. Hartnell, A. Tsicopoulos, A.J. Wardlaw, and A.B. Kay. 1991. Expression of mRNA and immunoreactivity for the granulocyte/macrophage colony-stimulating factor in activated human eosinophils. *J. Exp. Med.* 174, 749-752.

10. Levi-Schaffer, F., P. Lacy, N.J. Severs, T.M. Newman, J. North, B. Gomperts, A.B. Kay, and R. Moqbel. 1995. Association of granulocyte-macrophage colony-stimulating factor with the crystalloid granules of human eosinophils. *Blood.* 85, 2579-2586.

11. Ying, S., Q. Meng, L. Taborda-Barata, C.J. Corrigan, J. Barkans, B. Assoufi, R. Moqbel, S.R. Durham, and A.B. Kay. 1996. Human eosinophils express messenger RNA encoding RANTES and store and release biologically active RANTES protein. *Eur. J. Immunol.* 26, 70-76.

12. Lim, K.G., H.C. Wan, P.T. Bozza, M.B. Resnick, D.T. Wong, W.W. Cruikshank, H. Kornfeld, D.M. Center, and P.F. Weller. 1996. Human eosinophils elaborate the lymphocyte chemoattractants: IL- 16 (lymphocyte chemoattractant factor) and RANTES. *J. Immunol.* 156, 2566-2570.

13. Chihara, J., H. Oyamada, H. Yamada, A. Tsuda, Y. Kamada, and O. Urayama. 1997. Expression of mRNA for RANTES in human eosinophils. *Int. Arch. Allergy Immunol.* 114, 33-35.

14. A.R. Anwar, R. Moqbel, G.M. Walsh, A.B. Kay, and A.J. Wardlaw. 1993. Adhesion to fibronectin prolongs eosinophil survival. *J. Exp. Med.* 177, 839-843.

15. Baggiolini, M., and C.A. Dahinden. 1994. CC chemokines in allergic inflammation. *Immunol. Today.* 15, 127-133.

16. Kameyoshi, Y., A. Dorschner, A.I. Mallet, E. Christophers, and J.M. Schroder. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* 176, 587-592.

17. Schall, T.J., K. Bacon, K.J. Toy, and D.V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature.* 347, 669-671.

18. Taub, D., J. Dastych, N. Inamura, J. Upton, D. Kelvin, D. Metcalfe, and J. Oppenheim. 1995. Bone marrow-derived murine mast cells migrate, but do not degranulate, in response to chemokines. *J. Immunol.* 154, 2393-2361.

19. Devergne, O., A. Marfaing-Koka, T.J. Schall, M.B. Leger-Ravet, M. Sadick, M. Peuchmaur, M.C. Crevon, and K.J. Kim. 1994. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. *J. Exp. Med.* 179, 1689-1694.

20. Rathanaswami, P., M. Hachicha, M. Sadick, T.J. Schall, and S.R. McColl. 1993. Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts. Differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. *J. Biol. Chem.* 268, 5834-5839.

21. Marfaing-Koka, A., O. Devergne, G. Gorgone, A. Portier, T.J. Schall, P. Galanaud, and D. Emilie. 1995. Regulation of the production of the RANTES

chemokine by endothelial cells. Synergistic induction by IFN-γ plus TNF-α and inhibition by IL-4 and IL-13. *J. Immunol.* 154, 1870-1878.

22. Kwon, O.J., P.J. Jose, R.A. Robbins, T.J. Schall, T.J. Williams, and P.J.Barnes. 1995. Glucocorticoid inhibition of RANTES expression in human lung epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 12, 488-496.
23. Selvan, R.S., J.H. Butterfield, and M.S. Krangel. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. *J. Biol. Chem.* 269, 13893-13898.
24. Beck, L.A., S. Dalke, K.M. Leiferman, C.A. Bickel, R. Hamilton, H. Rosen, B.S. Bochner, and R.P. Schleimer. 1997. Cutaneous injection of RANTES causes eosinophil recruitment: comparison of nonallergic and allergic human subjects. *J. Immunol.* 159, 2962-2972.
25. Berkman, N., V.L. Krishnan, T. Gilbey, R. Newton, B. O'Connor, P.J. Barnes, and K.F. Chung. 1996. Expression of RANTES mRNA and protein in airways of patients with mild asthma. *Am. J. Respir. Crit. Care Med.* 154, 1804-1811.
26. Fahy, J.V., D.J. Figueroa, H.H. Wong, J.T. Liu, and J.S. Abrams. 1997. Similar RANTES levels in healthy and asthmatic airways by immunoassay and in situ hybridization. *Am. J. Respir. Crit. Care Med.*155, 1095-1100.
27. Holgate, S.T., K.S. Bodey, A. Janezic, A.J. Frew, A.P. Kaplan, and L.M. Teran. 1997. Release of RANTES, MIP-1α, and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am. J. Respir. Crit. Care Med.* 156, 1377-1388.

28. Rajakulasingam, K., Q. Hamid, F. O'Brien F, E. Shotman, P.J. Jose, T.J. Williams, M. Jacobson, J. Barkans, and S.R. Durham. 1997. RANTES in human allergen-induced rhinitis: cellular source and relation to tissue eosinophilia. *Am. J. Respir. Crit. Care Med.* 155, 696-703.

29. Chuluyan, H.E., T.J. Schall, T. Yoshimura, and A.C. Issekutz. 1995. IL-1 activation of endothelium supports VLA-4 (CD49d/CD29)-mediated monocyte transendothelial migration to C5a, MIP-1α, RANTES, and PAF but inhibits migration to MCP-1: a regulatory role for endothelium-derived MCP-1. *J. Leukoc. Biol.* 58, 71-79.

30. Kuna, P., S.R. Reddigari, T.J. Schall, D. Rucinski, M.Y. Viksman, and A.P. Kaplan. 1992. RANTES, a monocyte and T lymphocyte chemotactic cytokine releases histamine from human basophils. J. Immunol. 149, 636-642.

31. Valerius, T., R. Repp, J.R. Kalden, and E. Platzer. 1990. Effects of IFN on human eosinophils in comparison with other cytokines. A novel class of eosinophil activators with delayed onset of action. *J. Immunol.* 145, 2950-2958.

32. Hartnell, A., A.B. Kay, and A.J. Wardlaw. 1992. IFN-gamma induces expression of FcγRIII (CD16) on human eosinophils. *J. Immunol.* 148, 1471-1478.

33. Hartnell, A., D.S. Robinson, A.B. Kay, and A.J. Wardlaw. 1993. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology.* 80, 281-286.

34. Hansel, T.T., I.J. De Vries, T. Iff, S. Rihs, M. Wandzilak, S. Betz, K. Blaser, and C. Walker. 1991. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J. Immunol. Methods.* 145, 105-110.

35. Cromwell, O., J.P. Bennett, I. Hide, A.B. Kay, and B.D. Gomperts. 1991. Mechanisms of granule enzyme secretion from permeabilized guinea pig eosinophils. Dependence on Ca^{2+} and guanine nucleotides. *J. Immunol.* 147, 1905-1911.

36. Mahmudi-Azer, S., P. Lacy, B. Bablitz, and R. Moqbel. 1998. Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *J. Immunol. Methods.* 217, 113-119.

37. Bach, M.K., J.R. Brashler, E.N. Petzold, and M.E. Sanders. 1992. Superoxide production by human eosinophils can be inhibited in an agonist-selective manner. *Agents Actions* 35, 1-11.

38. Bach, M.K., J.R. Brashler, M.E. Sanders, and M.J. Bienkowski. 1991. Pitfalls in the quantitative estimation of the secretion of granule proteins by eosinophils. *J. Immunol. Methods.* 142, 243-250.

39. Menegazzi, R., G. Zabucchi, A. Knowles, R. Cramer, and P. Patriarca. 1992. A new, one-step assay on whole cell suspensions for peroxidase secretion by human neutrophils and eosinophils. *J. Leukoc. Biol.* 52, 619-624.

40. Boehm, U., T. Klamp, M. Groot, and J.C. Howard. 1997. Cellular responses to interferon-γ. *Annu. Rev. Immunol.* 15, 749-795.

41. Lacy, P., N. Thompson, M. Tian, R. Solari, I. Hide, T.M. Newman, and B.D. Gomperts. 1995. A survey of GTP-binding proteins and other potential key

regulators of exocytotic secretion in eosinophils. Apparent absence of rab3 and vesicle fusion protein homologues. *J. Cell. Sci.* 108, 3547-3556.

42. Fernvik, E., G. Hallden, J. Hed, and J. Lundahl. 1995. Intracellular and surface distribution of CD9 in human eosinophils. *APMIS* 103, 699-706.
43. Evans, W.H. 1987. Biological Membranes: A Practical Approach. Oxford, UK, IRL Press.
44. Dvorak, A. 1994. Ultrastructural studies on mechanisms of human eosinophil activation and secretion. *In* Eosinophils in Allergy and Inflammation. G.J. Gleich, and A.B. Kay eds. New York, Marcel Dekker, Inc. p159.
45. Gilat, D., R. Hershkoviz, Y.A. Mekori, I. Vlodavsky, and O. Lider. 1994. Regulation of adhesion of CD4$^+$ T lymphocytes to intact or heparinase- treated subendothelial extracellular matrix by diffusible or anchored RANTES and MIP-1β. *J. Immunol.* 153, 4899-4906.
46. Capron, M., M. Tomassini, G. Torpier, J.P. Kusnierz, S. MacDonald, and A. Capron. 1989. Selectivity of mediators released by eosinophils. *Int. Arch. Allergy. Appl. Immunol.* 88, 54-58.
47. Nüsse, O., M. Lindau, O. Cromwell, A.B. Kay, and B.D. Gomperts. 1990. Intracellular application of guanosine-5'-*O*-(3-thiotriphosphate) induces exocytotic granule fusion in guinea pig eosinophils. *J. Exp. Med.* 171, 775-786.
48. Fujisawa, T., S. Fukuda, J. Atsuta, R. Ichimi, H. Kamiya, and M. Sakurai. 1994. Interferon-γ induces interleukin-3 release from peripheral blood eosinophils. *Int. Arch. Allergy. Immunol.* 104, 41-43.

49. Ishihara, C., K. Ochiai, M. Kagami, H. Takashahi, G. Matsuyama, S. Yoshida, H. Tomioka, and N. Koya. 1997. Human peripheral eosinophils express functional interferon-γ receptors (IFN-γR). *Clin. Exp. Immunol.* 110, 524-529.

50. Sehmi, R., O. Cromwell, A.J. Wardlaw, R. Moqbel, and A.B. Kay. 1993. Interleukin-8 is a chemo-attractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. *Clinical and Experimental Allergy.* 23, 1027-1036.

51. Mosmann, T.R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today.* 17, 138-146.

52. Romagnani, S. 1991. Human Th1 and Th2 subsets: doubt no more. *Immunolo. Today.* 12, 256-257.

53. Lee, N.A., M.P. McGarry, K.A. Larson, M.A. Horton, A.B. Kristensen, and J.J. Lee. 1997. Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J. Immunol.* 158, 1332-1344.

54. Foster, P.S., S.P. Hogan, A.J. Ramsay, K.I. Matthaei, and I.G. Young. 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183, 195-201.

55. Pelus, L.M., O.G. Ottmann, and K.H. Nocka. 1988. Synergistic inhibition of human marrow granulocyte-macrophage progenitor cells by prostaglandin E and recombinant interferon-α, -β, and -γ and an effect mediated by tumor necrosis factor. *J. Immunol.* 140, 479-484.

56. Saito, H., T. Hayakawa, H. Mita, Y.S. Yui, and T. Shida. 1988. Augmentation of leukotriene C_4 production by gamma interferon in leukocytes challenged with an allergen. *Int. Arch. Allergy Appl. Immunol.* 87, 286-293.

57. Corrigan, C.J., and A.B. Kay. 1990. CD4 T-lymphocyte activation in acute severe asthma. Relationship to disease severity and atopic status. *Am. Rev. Respir. Dis.* 141, 970-977.

58. Fraenkel, D.J., P.G. Bardin, G. Sanderson, F. Lampe, S.L. Johnston, and S.T. Holgate. 1995. Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects. *Am. J. Respir. Crit. Care Med.* 151, 879-886.

59. Dubucquoi, S., P. Desreumaux, A. Janin, O. Klein, M. Goldman, J. Tavernier, A. Capron, and M. Capron. 1994. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J. Exp. Med.* 179, 703-708.

60. Beil, W.J., P.F. Weller, D.M. Tzizik, S.J. Galli, and A. M. Dvorak. 1993. Ultrastructural immunogold localization of tumor necrosis factor-alpha to the matrix compartment of eosinophil secondary granules in patients with idiopathic hypereosinophilic syndrome. *J. Histochem. Cytochem.* 41, 1611-1615.

Chapter 4

The association of the tetraspanin, CD63, with agonist-induced exocytosis in eosinophils from asthmatic subjects[1]

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Introduction

Eosinophils are major effector cells in allergic inflammation, asthma and host defense against parasitic helminths (1-4). They synthesize, store and release a wide range of pro-inflammatory mediators including at least four cationic proteins (1,2) and up to 23 cytokines, and growth factors (5,6). Eosinophils contain different populations of mediator-storage organelles including small secretory vesicles as well as crystalloid granules. The latter secretory granules are the site of storage of cytotoxic cationic proteins as well as a number of cytokines, chemokines and growth factors (7,8). The membrane-bound crystalloid granule is comprised of two compartments: an electron-dense crystalline core (internum) where major basic protein (MBP) (9,10) is stored and an electron-lucent matrix (7) where three cationic proteins (1,9) together with a number of other cytokines including IL-6 (11), and RANTES (12) are stored.

In eosinophils, three mediator-release mechanisms have been described; cytolysis or necrotic release, compound exocytosis, and piecemeal degranulation (PMD). In compound exocytosis, a number of granules fuse intracellularly to form a large degranulation chamber or cavity, which in turn fuses with the cell membrane before discharging its contents to extracellular space. In physiological conditions a more commonly seen mode of exocytotic mediator release in eosinophils is PMD whereby stored mediators are selectively released from intragranular pool leaving portions or all of the granules empty in the intact cell (13, 14). Various stimuli, including crosslinking of different subclasses of immunoglobulins are known to induce selective mediator release from eosinophils (15,16). Our own previous studies showed that IFNγ induces a rapid and selective intracellular mobilization and release of RANTES as early as 10 minutes after stimulation, while not affecting MBP translocation (12). The mechanisms underlying the selective mediator release in eosinophils remain unexplained. In fact, the molecular basis of PMD has not yet been identified.

CD63, also known as lysosome-associated membrane protein (lamp3) (17,18), is a member of the tetraspanin superfamily (TM4) whose membership has grown to 20 proteins since its discovery in 1990. Tetraspanins are membrane-associated molecules, which span the membrane four times (TM1-TM4) with two extracellular domains (EC1-EC2). As cell-surface proteins, tetraspanins appear to act as "molecular facilitators" by increasing the formation and stability of functional signaling complexes (19). CD63 is proposed to be involved in a number of cellular functions including cell activation (20) mediator release (21), adhesion (22), proliferation and differentiation (23). Originally identified as a platelet-activating antigen (24), CD63 is present in a wide variety of cells. In neutrophils and basophils, CD63 is an activation marker, in that its surface expression is upregulated from intracellular stores by stimulation (25,26). Present only in myeloperoxidase (MPO)-containing granules of neutrophils, CD63 has been described as a marker for azurophil granule fusion with the plasma membrane both *in vitro* and *in vivo* (19,25). In the rat basophilic leukemia cell line (RBL), an antibody against CD63 (AD1) inhibited IgE-mediated histamine release, which suggested a role for CD63 in mediator release (27).

Although, CD63 has been described in human eosinophils (28,29), very little is known about its possible function(s). Here we hypothesized that tetraspanin CD63 is involved in intracellular events associated with peripheral blood eosinophils activation and selective mediator mobilization and release from crystalloid granules. We have used

RT-PCR, immunofluorescent staining, confocal laser scanning microscopy, flow cytometry and β-hexosaminidase release assay to determine the expression, localization, and potential function of CD63 in peripheral blood eosinophils. Our findings indicate that CD63 may be an important element in the complex process of eosinophil activation and exocytosis, particularly PMD.

Materials and Methods

Eosinophil Purification

Eosinophils were purified from peripheral blood of documented mild atopic asthmatic subjects as previously described (11,12,30). Briefly, samples of peripheral blood (100 ml) were collected in heparin-containing tubes. Erythrocytes were sedimented for 45 min at room temperature with 10 ml 5% dextran (100,000-200,000 kDa; Sigma, Oakville, ON, Canada). The upper phase of the leukocyte-rich plasma was then layered onto a 15 ml Ficoll gradient (Pharmacia Biotech, Inc, Uppsala, Sweden) and centrifuged for 25 min at 1000*g*. After removal of excess plasma, mononuclear layer, and Ficoll, the resulting granulocyte pellet was resuspended in 2 ml RPMI-1640 (BioWhittaker, Walkersville, MD). Contaminating erythrocytes were removed by hypotonic lysis on ice in 1 ml sterile H_2O for 5 seconds. The resultant pellet was incubated with a mixture of 12 μl anti-CD16, 10 μl anti-CD3 and 10 μl anti-CD14-coated immunomagnetic beads (MACS beads; Miltenyi-Biotec, Bergisch-Gladbach, Germany) for 45 min at 4°C to remove contaminating neutrophils, lymphocytes, and monocytes respectively by negative selection on a magnetic column. The resulting eosinophil purity was usually greater than 99%.

Granule purification

Purified eosinophils (4×10^7) were washed in 10 ml ice cold buffer A (10 mM HEPES, 0.25 M sucrose, 1mM EGTA, pH 7.4) and resuspended in ice cold homogenization buffer (HEPES-buffered sucrose supplemented with 2 mM/L $MgCl_2$ and 1 mM ATP and 5 µg/mL each of leupeptin, aprotinin, and TAME, pH 7.4) to optimal subfractionation density (between 10 and 15×10^6/mL). Cells then were subjected to 15 to 20 passes through a ball-bearing homogenizer (EMBL, Heidelberg, Germany) possessing 12-µm clearance. To obtain the postnuclear supernatant the homogenate was centrifuged at 400 *g* for 10 min. The resulting postnuclear supernatant was subjected to density centrifugation at 10,000*g* for 15 min at 4°C. Supernatant containing light membrane was discarded and the pellet containing crystalloid granules was resuspended in 50 µl buffer B and used in experimental procedures immediately after.

RT-PCR analysis

Total RNA was extracted from resting and stimulated eosinophils using the QIAGEN QIAShredder and RNeasy MiniKit (QIAGEN Inc, Mississauga, ON, Canada) according to the manufacturer's instruction. Three different stimuli were used to stimulate the eosinophils (2×10^6 cells per each of the following time- points: 0, 2, 4 and 16 hours), IFNγ (20 ng/ml, R & D Systems, Minneapolis, Minnesota, USA), a combination of C5a (800 nM) and cytochalasin B (CB), (10 ng/ml, Sigma), and a cocktail of IL-3 (10 ng/ml), IL-5 (5 ng/ml) and GM-CSF (10 ng/ml) (Genzyme, Cambridge, Ms).

One third of each sample of total cellular RNA (~300 ng) were reverse transcribed by incubating with 200 U superscript II reverse transcriptase (Gibco BRL Life Technologies, Ltd, Grand Island, NY), 1X first Strand Buffer, and 0.5 mM of mixed dNTPs (Gibco BRL). Aliquots (2 μl) of cDNA were used in a 20 μl PCR reaction containing 2.5 U of *Taq* DNA polymerase, 1X PCR buffer, 25 μM mixed dNTPs (Gibco BRL), and 0.2 μM each of the appropriate forward (5′) and reverse (3′) primers. A hot start was performed in the initial denaturation step at 95°C for 3 min before samples were subjected to 35 cycles of heating to 95°C for 45 sec, 55°C for 1 min, and 72°C for 30 sec in a thermal cycler (M-J Research, Watertown, MA). A final extension step was then performed by heating to 72°C for 7 min. The following PCR primers (synthesized by the DNA services laboratory, University of Alberta) were used for the CD63 amplification, 5′-AACAACCACACTGCTTCGATCC-3′ (forward) and 5′-TTTCGG TCTGAAAAAATAATCCGTTT -3′ (reverse) (CD63 product of 427 bp). Amplification of β2-microglobulin (5′-CTCGCGCTACTCTCTCTTTCTGG-3′, 5′-GCTTACATGTCT CGATCCGACTTAA-3′) was used to verify that equal amounts of RNA were used for reverse transcription and PCR amplifications from the different experimental conditions. The PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The densities of RT-PCR bands were determined by scanning the photographs and using ImageMaster computer software (Pharmacia).

Flow cytometry

Eosinophils (5×10^5 cells per test) incubated in the presence or absence of IFNγ, C5a/CB and IL-3/IL-5/GM-CSF (10 min) were fixed in 5% formalin (10 min at 4°C) and blocked in 5% milk in flow buffer (PBS + 1% BSA + 0.1% sodium azide) overnight at 4°C. Following blocking, cells were washed (X3) in flow buffer and incubated with 5 μg/ml of one of the following antibodies: mouse monoclonal anti-CD63 (IgG_1;

Pharmingen, San Diego, CA), or mouse IgG$_1$ isotype control (R & D Systems), for 60 min on ice. After three washing steps cells were incubated with goat Fab$_2$ anti-mouse IgG conjugated to phycoerythrin (5 μg/ml) (Cedarlane Laboratories Ltd, Hornby, ON, Canada) for 30 min at 4°C. Cells were subsequently washed three times and resuspended in flow buffer to a final density of 1×10^6 cells/ml and analyzed on a FACScan instrument using CellQuest software (Becton Dickinson, San Jose, CA). Eosinophil crystalloid granules obtained from purified eosinophils were subjected to the same procedure described above, and further examined using FACS analysis. To examine the intracellular or intragranular pool of CD63, eosinophils or their purified granules were permeabilized by adding 0.1% saponin to the blocking reagent.

Immunofluorescent labeling

Cytospins of resting as well as IFNγ-, C5a/CB- and IL-3/IL-5/GM-CSF-stimulated (10 min) eosinophils were prepared by spinning 3×10^4 cells (suspended in 100 μl 20% FCS in RPMI-1640) in a Cytospin 2 centrifuge (Shandon, UK) at 800 rpm for 2 min. To prepare cytospins of crystalloid granules the same procedure was carried out on highly purified crystalloid granules. Slides were then foil-wrapped and stored at -20°C until used. Slides of resting and agonist-stimulated eosinophils were fixed for 8 min in 2% paraformaldehyde in PBS (room temperature) and washed (X5) in Tris-buffered saline (TBS, pH 7.4). Following fixation, cytospins were blocked using 3% FCS in a humidified container (30 min). After a second washing step, slides were incubated overnight with TBS containing 1% mouse monoclonal anti-human CD63 (5 μg/ml) at 4°C. BODIPY-FL conjugated secondary antibody (20 μg/mL) (Molecular Probes, Eugene, OR, USA) was used to detect immunoreactivity of CD63 (2h, room temperature). Following another washing step, slides were blocked again for 2 h using goat anti-mouse IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), (50 µg/mL) and double-labeled with either mouse monoclonal anti-human MBP (1%) (BMK-13, generated in-house (31) or mouse monoclonal anti-human RANTES (5 µg/mL) (R&D system) at 4°C. Immunoreactivity against MBP and RANTES were detected by incubating slides with15 µg/mL Rhodamine (TRITC)-labeled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for 2 h. Mouse IgG1 (R&D Systems) at equivalent concentrations was used as the isotype control. After a final washing step, 10 µl of the anti-bleaching agent, 0.4% n-propyl gallate (Sigma) in 3:1 glycerol: 10xTBS, was applied to the slides before coverslip attachment.

Confocal laser scanning microscopy

Immunofluorescent staining of resting and stimulated eosinophils as well as purified crystalloid granules were examined using a Zeiss Laser Scanning Confocal Microscope (LSM 510) mounted on a Zeiss Axiovert M100 inverted microscope with a 63X plan-apochromatic lens. The 488nm laser line (generated from 25mW argon laser) and 543nm laser line (generated from 1mW HeNe laser) were used to image BODIPY-FL (green) and Rhodamine (TRITC) (red), used in the experiments. A band pass filter (505-550 nm) was used to collect emission from BODIPY-FL and a long pass filter (560nm) was used to collect signals from TRITC. To avoid spillover of the fluorochromes, sequential scanning mode of the machine was used to collect images from double stained samples. Image acquisition was optimized using the required pinhole setting, photomultiplier gain, and offset. Higher spatial resolution was achieved by using the appropriate zoom on the computer. Images were further analyzed and developed using LSMIB 4.0 software.

Measurement of β-hexosaminidase release and statistical analysis

Freshly purified eosinophils (2×10^5 cells) were stimulated using IFNγ, C5a/CB and IL-3/IL-5/GM-CSF for 10 min. Cell free supernatants (50µl) were mixed with 50µl substrate solution (1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide in 0.2 M citrate buffer, pH 4.5, and 0.1% Triton X-100) and incubated for 60 min at 37°C. The reaction was terminated by the addition of 150 µl ice-cold 0.2 M Tris, and the fluorescence (excitation 360 nm, emission 460 nm) was measured in a Millipore CytoFluor 2350 plate reader (Millipore, Nepean, ON, Canada) as described in earlier studies (11,12). Experiments were done in triplicates. Values were averaged and their standard error of the mean was calculated. Results were analyzed for significance using Student's t test and they were considered significant when $p < 0.01$.

Results

CD63 mRNA expression in human eosinophils

Highly purified peripheral blood eosinophils were examined by RT-PCR to detect the CD63 mRNA. Our results showed that both resting and IFNγ (20 ng/ml) stimulated eosinophils express CD63 mRNA. No detectable changes in mRNA expression was evident after IFNγ stimulation for 2, 4 and 16h (fig. 1). The same was true for eosinophils stimulated by C5a/CB, or IL-3/IL-5/GM-CSF stimulation (data not shown).

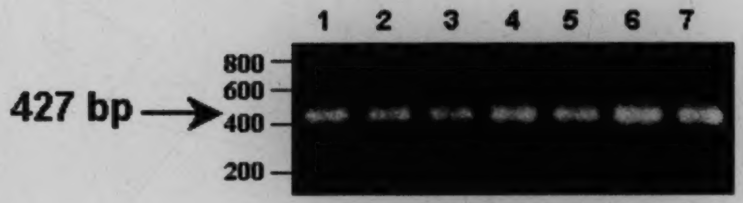

Fig. 1.4. RT-PCR analysis of eosinophil CD63 mRNA expression:

Lane1= resting eosinophils, lane 2 = 2h after IFNγ stimulation, lane 3 = 2h medium alone, lane 4 = 4h after IFNγ stimulation, lane 5 = 4h medium alone, lane 6 = 16h after IFNγ stimulation, lane 7= 16h medium alone. Resting peripheral blood eosinophils express CD63 mRNA (lane 1). Neither IFNγ (lane 2, 4 & 6), nor C5a/CB or IL-3/IL-5/GM-CSF (date not shown) stimulation of eosinophils showed significant regulatory effect on CD63 mRNA expression.

CD63 protein expression and localization in human eosinophils

CD63 protein expression was examined using a combination of immunofluorescent staining and confocal laser scanning microscopy on cytospins of purified peripheral blood eosinophils. Cytospins of freshly purified eosinophils were prepared and immunostained using monoclonal anti-human CD63. CD63 was expressed in resting peripheral blood eosinophils, which appeared predominantly localized to the membrane of crystalloid granules (fig. 2A). The association of immunoreactivity against CD63 with crystalloid granules membrane was further confirmed by immunofluorescent staining of an isolated population of crystalloid granules (fig. 2B).

To determine the intracellular localization site of CD63 relative to MBP (marker for eosinophils granule crystalline cores) and RANTES (marker for granule matrix regions), we carried out double-immunofluorescent staining procedure on a population of isolated granules as well as freshly purified eosinophils. While immunoreactivity against MBP predominantly localized to the core region (fig. 2C-D) and did not colocalize with CD63, the immunoreactivity against RANTES and CD63 colocalized to the peripheral compartment of the crystalloid granules highlighted by yellow color in the combined image (fig. 2E).

Localization and mobilization of CD63 in stimulated eosinophils

To examine the intracellular localization and kinetics of CD63 mobilization relative to MBP in agonist-stimulated cells, freshly isolated eosinophils were stimulated (10 min) with IFNγ (20 ng/ml), C5a (800 nM) /CB (10 ng/ml) or IL-3 (10 ng/ml) /IL-5/ (5 ng/ml) GM-CSF (10 ng/ml). Cytospins of stimulated eosinophils were prepared, fixed, double immunostained for CD63 and MBP and examined under confocal fluorescent microscopy. As early as 10 min after IFNγ (fig. 2 F-H) or C5a/CB stimulation (fig. 2 I-K), CD63 immunostaining appeared to be confined only to the granules adjacent to the cell membrane while MBP immunoreactivity remained relatively unaltered. In contrast to IFNγ and C5a/CB, stimulation with IL-3/IL-5/GM-CSF induced the appearance of discrete clusters of CD63 that colocalized predominantly with eosinophil MBP (fig. M-O). IFNγ, and C5a/CB-induced intracellular mobilization of CD63, were inhibited by the tyrosine kinase inhibitor, genistein (10^{-6} M) and the same was true for IL-3/IL-5/GM-CSF-induced translocation of CD63 and MBP (fig. 2 P) (n=5).

CD63 translocation coincided with that of RANTES

We have previously shown that IFNγ stimulation of eosinophils induces the rapid mobilization of RANTES to cell periphery prior to its release to extracellular space (12). To examine the association of CD63 with RANTES translocation to cell periphery, double-immunofluorescent staining with specific antibodies to RANTES and CD63 was conducted on IFNγ-stimulated eosinophils. Following IFNγ stimulation (10 min) of eosinophils, immunoreactivity against RANTES colocalized with that of CD63, with both translocating to the periphery of the cells (fig. 2 Q-S). The presence of CD63- and MBP-negative granules in stimulated eosinophils was further confirmed by differential interference contrast (DIC) image (T). Mouse IgG1 at equivalent concentrations was used as isotype control for all the above mentioned immunostaining procedures (fig. 2L)

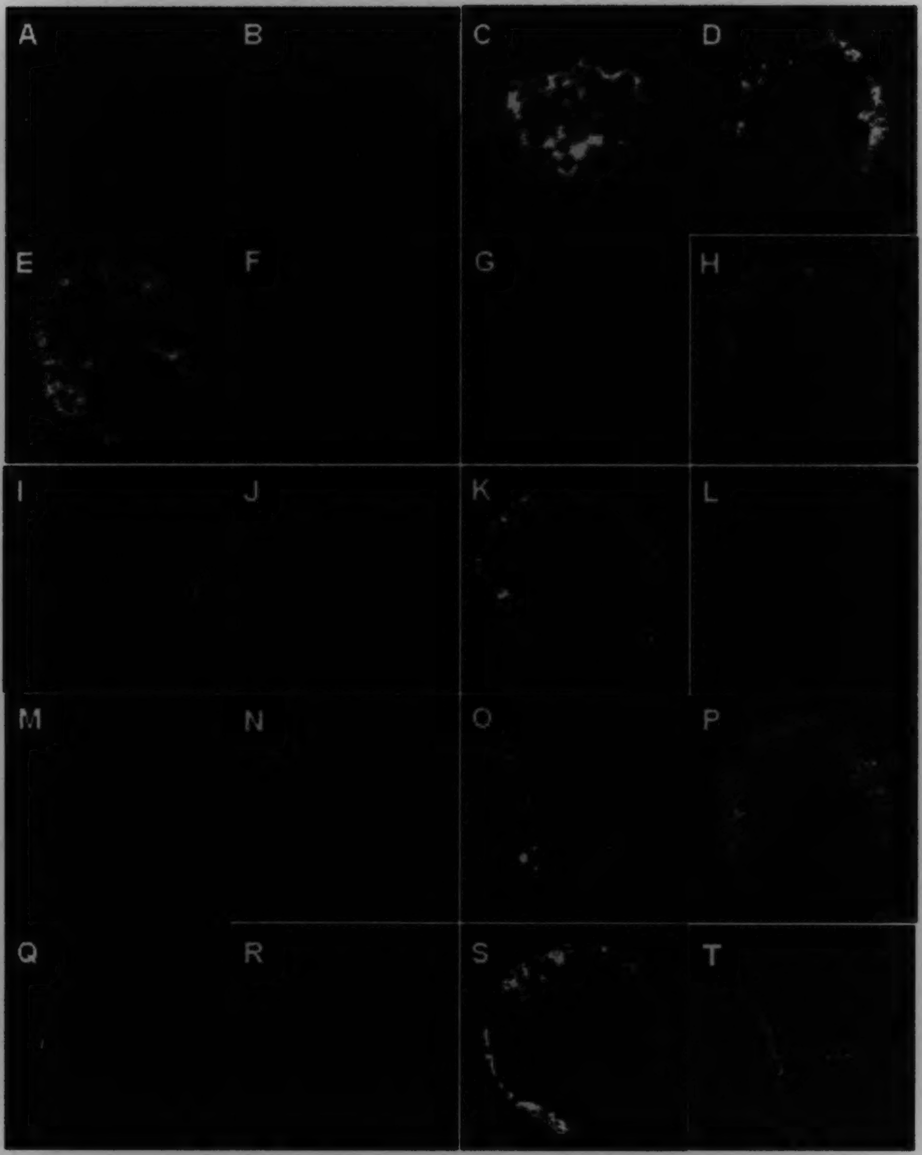
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Fig. 2.4. Confocal laser scanning microscopy (CLSM) images of immunostained peripheral blood eosinophils of asthmatic subjects: Representative images of human eosinophils stained with TRITC-conjugated secondary antibody (red color) to detect the MBP as well as RANTES immunoreactivity, or BODIPY-FL-conjugated secondary antibody (green color)to detect immunoreactivity against CD63. (A) Resting peripheral blood eosinophils labeled with BODIPY-FL indicating CD63 immunoreactivity. (B) Highly purified crystalloid granules immunostained with anti-CD63. (C) Higher magnification of a combined image of eosinophil crystalloid granules immunostained for MBP (TRITC) indicating core-associated immunoreactivity, and CD63 indicating matrix-associated CD63 immunoreactivity. (D) Combined image of double immunofluorescence (MBP & CD63) staining of resting eosinophils, red TRITC represents immunoreactivity against MBP and green BODIPY-FL is indicative of CD63 immunoreactivity. (E) Combined image of double immunofluorescence (RANTES & CD63) staining of resting eosinophils, TRITC detected RANTES and BODIPY-FL detected CD63. (F-H) Double immunofluorescence (MBP & CD63) staining of IFNγ-stimulated eosinophils (time=10 min): MBP immunostaining (F), CD63 immunostaining (G) and combined image (H). (I-K) Double immunofluorescence (MBP & CD63) staining of C5a/CB-stimulated eosinophils (time=10 min): MBP immunostaining (I), CD63 immunostaining (J) and combined image (K). (L) Combined image of isotype control for CD63 and MBP immunostaining. (M-O) Double immunofluorescence (MBP & CD63) staining of IL-3/IL-5/GM-CSF-stimulated eosinophils (time=10 min): MBP immunostaining (M), CD63 immunostaining (N) and combined image (O). (P) Combined image of double immunofluorescence (MBP & CD63) staining of IFNγ-stimulated eosinophils pre-incubated with genistein (10^{-6} M), which inhibited the IFNγ-induced translocation of CD63. (Q-T) Double immunofluorescence (RANTES & CD63) staining of IFNγ-stimulated eosinophils: RANTES immunostaining (Q), CD63 immunostaining (R), combined image (S), and differential interference contrast (DIC) image of the same cell (T). Original magnification is 63×10 X for all images.

Dexamethasone effect on agonist-induced CD63 translocation

To examine the effect of dexamethasone on agonist-induced intracellular translocation of CD63, freshly purified eosinophils were incubated in the presence of dexamethasone (10^{-6} M) for 60 min prior to agonist stimulation. Interestingly, dexamethasone inhibited the IFNγ (fig 3A-D), C5a/CB (fig 3E-H) or IL-3 /IL-5/GM-CSF (fig 3I-L)-induced intracellular mobilization of CD63 (n=6).

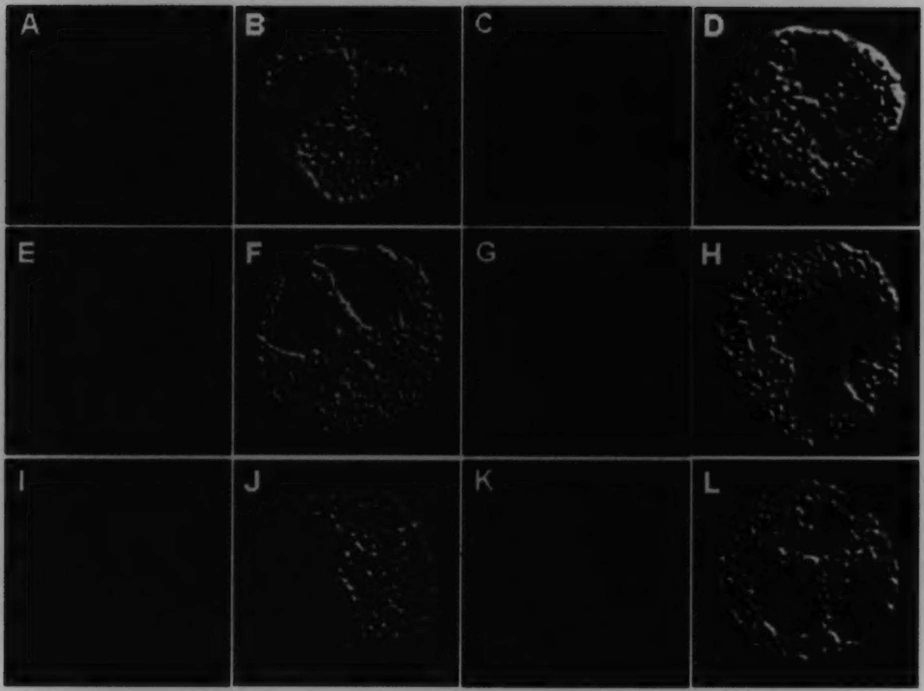
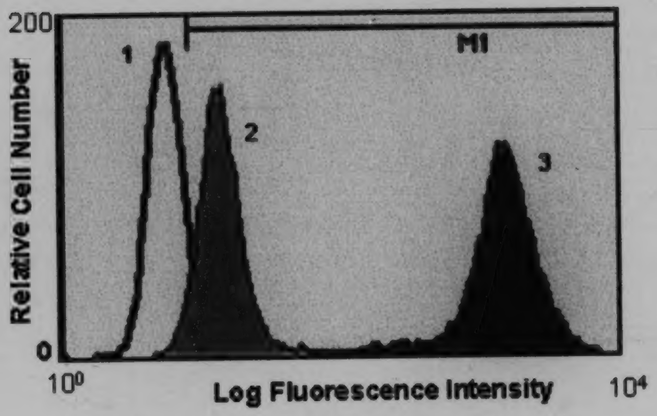


Fig. 3.4 The effects of dexamethasone on agonist-induced CD63 translocation: Representative images of human eosinophils single stained with BODIPY-FL-conjugated secondary antibody (green color) to detect immunoreactivity against CD63. (A, B) immunofluorescence (CD63) staining of IFNγ-stimulated eosinophils and DIC image of the same cell (time=10 min). (C, D) Immunofluorescence staining of eosinophils pre-incubated with dexamethasone (10^{-6} M) prior to IFNγ-stimulation, and the DIC image of the same cell. (E, F) Immunofluorescence staining of C5a/CB-stimulated eosinophils and DIC image of the same cell. (G, H) Immunofluorescence staining of eosinophils pre-incubated with dexamethasone (10^{-6} M) prior to C5a/CB-stimulation, and the DIC image of the same cell. (I, J) Immunofluorescence staining of IL-3/IL-5/GM-CSF stimulated eosinophils and DIC image of the same cell. (K, L)) Immunofluorescence staining of

eosinophils pre-incubated with dexamethasone (10^{-6} M) prior to IL-3/IL-5/GM-CSF - stimulation, and the DIC image of the same cell. Original magnification is 63×10 X for all images.

CD63 surface expression in resting and stimulated eosinophils

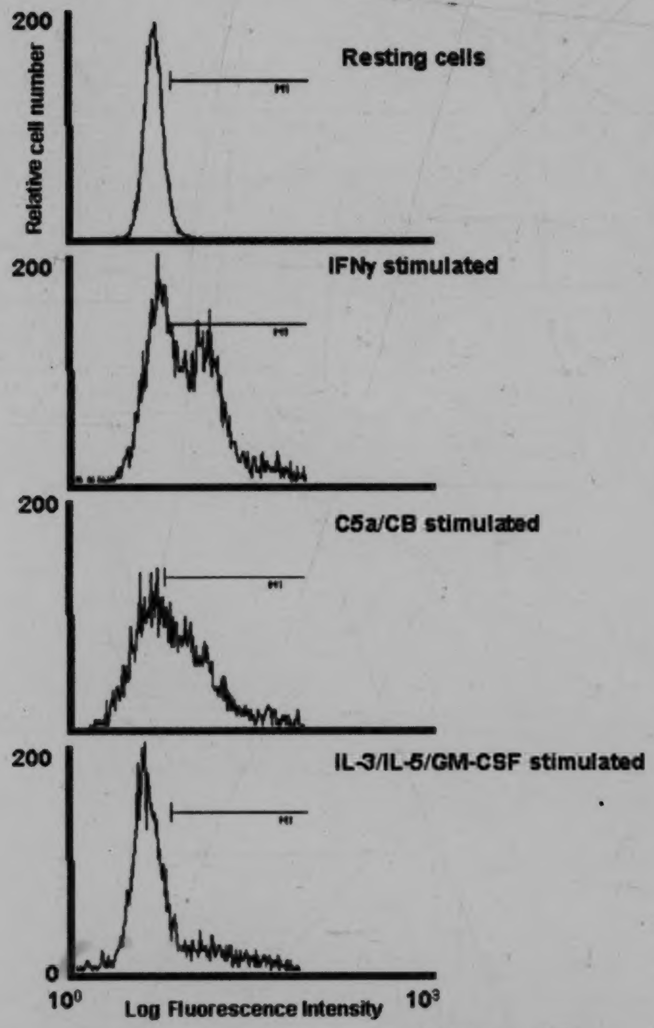
The surface expression of CD63 was examined by flow cytometry. Freshly isolated eosinophils were immunostained with anti-CD63 antibody and analyzed using FACScan instrument. Our results indicated that CD63 is expressed on the surface of resting eosinophils. Saponin-permeabilized cells showed a significant shift (50-fold) in mean fluorescent intensity (MFI), which may be indicative of a much larger intracellular site of CD63 expression and/or storage (fig. 4) (n=4).



	%Gated	MFI
1: Isotype control	4.91	4.67
2: Non-permeabilized cells	61.15	10.87
3: Permeabilized cells	99.68	514.92

Fig. 4.4. Analysis of CD63 surface expression: CD63 expression on the surface of purified eosinophils was examined using flow cytometry analysis. (1) Isotype control, (2) CD63 expression in non-permeabilized eosinophils, (3) CD63 expression in (0.1%) saponin -permeabilized eosinophils (3).

CD63 surface expression was further enhanced after agonist stimulation (10 min), with C5a/CB inducing maximum upregulation in contrast to the combination of IL-3/IL-5/GM-CSF, which induced minimum upregulation (fig. 5) (n=4). This surface upregulation of CD63 coincided with intracellular translocation of CD63 and RANTES upon agonist stimulation. Upregulation of CD63 surface expression upon stimulation with either agonist was equally inhibited by genistein (n=4).

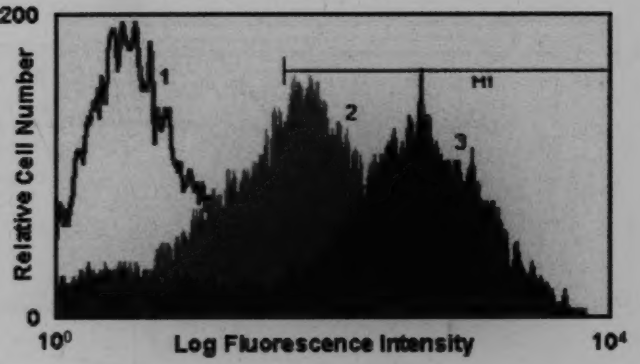


	%Gated	MFI
1: Resting cells	6.82	10.96
2: IFNγ stimulated cells	54.96	46.72
3: C5a/CB stimulated cells	63.87	49
4: IL-3/IL-5/GM-CSF stimulated cells	20.78	23.76

Fig. 5.4. Analysis of CD63 surface expression in agonist stimulated eosinophils: (1) CD63 surface expression on resting eosinophils, (2) CD63 surface expression on IFNγ-stimulated eosinophils, (3) CD63 surface expression on C5a/CB-stimulated eosinophils, (4)CD63 surface expression on IL-3/IL-5/GM-CSF-stimulated eosinophils.

CD63 expression on isolated populations of crystalloid granules

We studied the expression of CD63 on the surface of isolated crystalloid granules and examined the presence of an intragranular pool and/or an inverted membrane configuration of CD63. We immunostained both a permeabilized and a non-permeabilized populations of dispersed and highly purified eosinophil crystalloid granules. The obtained results indicated that CD63 was expressed on the surface of crystalloid granules. Interestingly, mean fluorescent intensity (MFI) of permeabilized granules was increased approximately 2-folds in saponin-permeabilized granules (fig. 6) (n=4). To ensure that granules were intact after isolation, granule preparations were immunostained with antibodies against MBP or RANTES and no immunostaining was detected by flow cytometry against either of the two mediators in non-permeabilized granule preparation. Only permeabilized granules showed MBP and RANTES immunoreactivity (data not shown).



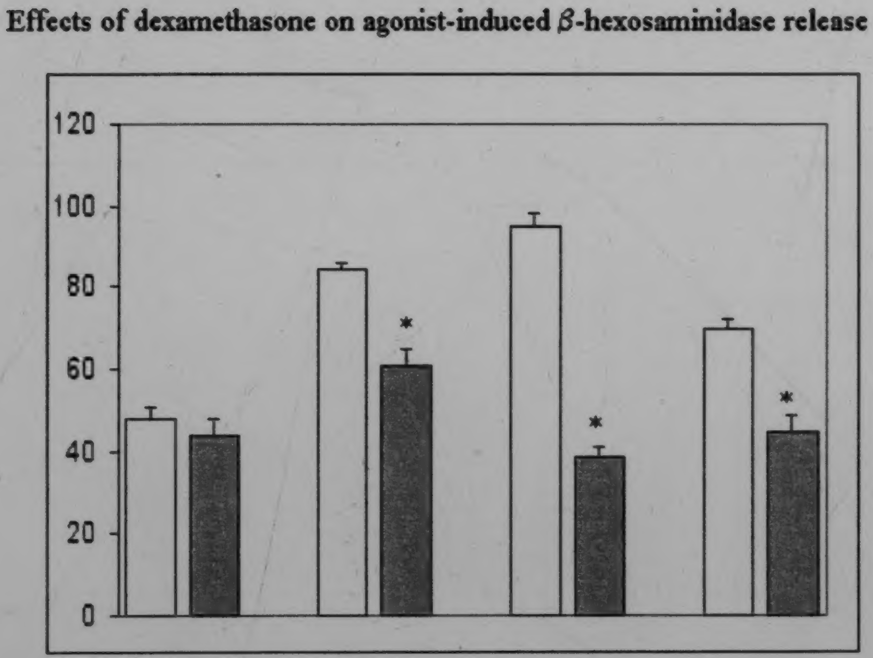
	%Gated	MFI
1: Isotype control	4.75	110.63
2: Non-permeabilized granules	42.16	277.95
3: Permeabilized granules	74.86	570.15

Fig. 6.4. Analysis of CD63 expression on the surface of purified crystalloid granules: (1) Isotype control, (2) CD63 expression on the surface of non-permeabilized purified crystalloid granules, (3) CD63 expression in saponin-permeabilized granules.

Mediator release in stimulated eosinophils and the effects of dexamethasone and the tyrosine kinase inhibitor, genistein

We examined the association between intracellular translocation and surface upregulation of CD63 with mediator release upon agonist-stimulation. We measured β-hexosaminidase release in the supernatant of IFNγ, C5a/CB or IL-3/IL-5/GM-CSF stimulated-eosinophils. Our results indicated that agonist stimulation of eosinophils (10 min) induced β-hexosaminidase release, with C5a/CB inducing maximum release in contrast to the combination of IL-3/IL-5/GM-CSF, which induced minimum release (n=6). β-hexosaminidase release occurred concurrently with CD63 translocation to the cell periphery and the cell surface upregulation. Indeed, β-hexosaminidase release induced by various stimuli was associated with agonist-induced enhancement of CD63 surface expression measured by mean fluorescent intensity. In particular, C5a/CB induced more β-hexosaminidase release in parallel with its stronger effect on CD63 surface expression (fig. 7 & fig.5).

Glucocorticoids have been long recognized for their anti-inflammatory effects. Indeed, glucocorticoids represent a mainstay of therapy for bronchial asthma, and other allergic diseases. The clinical efficacy of glucocorticoids in allergic inflammation is partially mediated by their potent inhibitory effects on the elaboration of pro-inflammatory and eosinophil-active cytokines (32). We examined the potential inhibitory effects of glucocorticoids on β-hexosaminidase release and CD63 translocation. Pre-treatment of eosinophils with dexamethasone (60 min) prior to agonist-activation, down-regulated β-hexosaminidase release significantly (fig. 7). Dexamethasone, in the absence of agonist stimulation, did not alter the β-hexosaminidase release. To examine the potential involvement of tyrosine kinases activity in CD63 translocation following agonist stimulation, eosinophils were pre-incubated with genistein. Genistein pre-treatment down-regulated the β-hexosaminidase release in agonist-activated eosinophils in a similar fashion to dexamethasone (data not shown).


Effects of dexamethasone on agonist-induced β-hexosaminidase release

RPMI	+	+	-	-	-	-	-	-
IFNγ	-	-	+	+	-	-	-	-
C5a/CB	-	-	-	-	+	+	-	-
IL-3/IL-5/ GM-CSF	-	-	-	-	-	-	+	+
Dex.	-	+	-	+	-	+	-	+

Fig. 7.4. Mediator (β-hexosaminidase) release in agonist stimulated eosinophils and effects of dexamethasone: Freshly purified eosinophils (2×10^5 cells) were stimulated using IFNγ, C5a/CB and IL-3/IL-5/GM-CSF, for 10 min and β-hexosaminidase was measured in cell free supernatants. The effect of dexamethasone on agonist-induced mediator release was examined by pre-incubating cells with dexamethasone (10^{-6}) for 60 min. The Student T-Test was used to analyze the results. Values represent averages of triplicate measurements. A similar trend of release was observed in 6 separate donors. The first column shows spontaneous release of β-

hexosaminidase from eosinophils, the second and third columns show IFNγ-induced release and dexamethasone effect, the fourth and fifth columns represent the C5a/CB-induced release and dexamethasone effect, and the sixth and seventh columns show the IL-3/IL-5/GM-CSF induced release and dexamethasone effect. * Represents the statistical significance of dexamethasone inhibition of mediator release, error bars represent the mean and SEM of measurements ($* \ p < 0.01$).

Discussion

In this study, we have investigated the expression, and localization of CD63 in resting and activated human peripheral blood eosinophils. We have further examined the association of CD63 intracellular localization and surface expression with selective mediator mobilization (RANTES) and release (β-hex) in agonist-activated eosinophils. We have previously examined RANTES ultrastructural localization in human eosinophils by immunogold (12).

Selective mediator release or piecemeal degranulation is a well documented mode of mediator release in human eosinophils with around 67% of all airway mucosal eosinophils exhibiting lucent granules indicative of PMD in association with asthma (33, 34, 35), allergic rhinitis (36, 37), and nasal polyposis (13, 38,39). In allergen-exposed nasal airways, virtually all viable eosinophils in mucosal tissue showed signs of PMD under active disease conditions (36). Eosinophil granule alterations reflecting PMD have also been described in guinea pig models of asthma (40, 41).

In a previous study we showed that selective release of RANTES in eosinophils upon IFNγ stimulation occurred in parallel with a rapid initial mobilization of RANTES to the cell periphery followed by secretion to the extracellular space. (12). RANTES trafficking as well as release was suggested to be associated with a pool of rapidly mobilizable small mediator-containing vesicles. These vesicles are thought to bud from crystalloid granules, fuse with plasma membrane and selectively evacuate the content of specific granules to extracellular space (42, 43). However, no molecular and cell surface marker for this gradual and selective mediator release (PMD) has been identified. To date, researchers have only relied on determining the percentage of altered granules (granules exhibiting ultrastructural signs of protein release) in tissue eosinophils (13).

In this study we propose CD63 as a molecular marker for PMD since it appears to be associated with intracellular mediator mobilization (RANTES) to the cell periphery and extracellular space (β-hex). In support of this proposal, we have demonstrated that in resting eosinophils, CD63 is expressed on the membrane of all MBP immunoreactive crystalloid granules. However, upon IFNγ or C5a/CB stimulation, CD63 appeared to localize to the periphery of granules that are adjacent to the plasma membrane. The CD63 translocation to the cell periphery upon IFNγ stimulation predominantly coincided with RANTES translocation, which has been shown also to mobilize to the cell periphery prior to its release to extracellular space (12).

Interestingly, agonist stimulation upregulated CD63 on the eosinophil cell surface. Agonist-induced surface upregulation of CD63 appeared to coincide with β-hexosaminidase release suggesting a potential role for CD63 in mediator release. Indeed, the agonist that induced higher upregulation of surface membrane expression for CD63 equally induced higher β-hexosaminidase release (fig. 5 & fig. 7). The surface upregulation of CD63 following cell stimulation and subsequent mediator release in eosinophils are in agreement with previous observations in neutrophils. These studies demonstrated CD63 mobilization to the plasma membrane following cell stimulation. CD63 in human neutrophils has been used as a marker for the fusion of azurophilic granules with plasma membrane (25). Surface upregulation of CD63 in eosinophils have been shown to occur *in vivo*, since surface expression of CD63 was observed to be upregulated in eosinophils recovered from bronchoalveolar lavage of allergic asthmatic patients (44).

In localization studies, we provided evidence that CD63 is expressed on the membrane of purified crystalloid granules. To control for structural integrity of purified granules and to show that CD63 immunostaining is not intragranular, we immunostained the same granule populations with specific antibody against MBP as well as RANTES. No immunoreactivity against MBP or RANTES was observed indicating that the dispersed granules were intact. Interestingly, following permeabilization, granules showed two fold increase in immunoreactivity against CD63. This increase in immunoreactivity of permeabilized granules is presumably caused either by intragranular storage of CD63 or by the presence of granule-membrane associated CD63 on both the inner and outer leaflet of the granule. However, it is unlikely that there will be an intragranular pool of CD63 approximately equal to the membrane-associated pool. We therefore, speculate that this two-fold increase in immunoreactivity of permeabilized granules may be due to the interaction with the same epitope of CD63 facing the inner leaflet of the granules, potentially caused by the dynamic fusion of small secretory vesicles with crystalloid granules. Indeed, a previous study showed that members of tetraspanin superfamily such as CD37, CD53, CD63, CD81 and CD82 are concentrated on the internal membrane of MHC class II-enriched compartment (45). This indicates that the "extracellular loops" of tetraspanins may face the inner comportment of an intracellular organelle.

Interestingly, and unlike IFNγ and C5a/CB, stimulation with IL-3/IL-5/GM-CSF induced the appearance of discrete clumps of CD63, which predominantly colocalized with large intracellular stores of MBP. The formation of such large intracellular pools of MBP may be associated with compound exocytosis. Furthermore, the colocalization of CD63 with MBP pools may indicate the association of CD63 with compound exocytosis induced by eosinophil-active cytokines. The association of CD63 with intracellular mediator mobilization (RANTES and MBP) and release (β-hex) may suggest a role for CD63 as a "molecular chaperone" and may be indicative of the association of CD63 with eosinophil secretory processes.

We used genistein to examine the specificity and the involvement of protein kinases in eosinophil response to agonist stimulation. Genistein is a broad specificity protein kinase inhibitor, shown to inhibit protein tyrosine kinases as well as other protein kinases by acting as a competitive inhibitor of ATP (46, 47). Tyrosine kinase activity has been implicated in eosinophil activation and mediator release. Genistein has been shown to inhibit tyrosine phosphorylation, cell activation, and mediator release responses of eosinophils induced by different agonists (48, 49,50,51). Genistein has been used to inhibit early steps in the IFNγ receptor-signaling pathway following ligand binding (52). In our hands, genistein fully inhibited agonist-induced (IFNγ, C5a/CB and IL-3/IL-5/GM-CSF cocktail), intracellular translocation and mobilization of CD63 and RANTES as well as cell surface upregulation of CD63 and β-hex release. The inhibitory effects of genistein on intracellular movement of CD63 indicated a functional involvement of protein kinases in these processes. Nevertheless, the precise signal transduction pathways that link agonist stimulation with CD63 translocation and mediator release remains unknown.

Dexamethasone also inhibited agonist-induced intracellular translocation and mobilization and cell surface upregulation of CD63, and at the same time it also down-regulated agonist-induced β-hexosaminidase release. These observations further support the association of CD63 mobilization and translocation to the cell periphery with agonist-induced β-hexosaminidase release. Eosinophils are known to be important targets of glucocorticoides effects (53). Disruption of the cytokine network by glucocorticoids is known to indirectly diminish eosinophil generation, survival, and function. A number of studies indicate the direct effect of glucocorticoids on the survival and other functions of these cells including activation, adhesion and apoptosis (54-56). Indeed, glucocorticoids have been shown to inhibit antibody-dependent cellular cytotoxicity and degranulation in rat eosinophils (57). An inhibitory effect for dexamethasone on agonist-induced IL-8 and MCP-1 generation as well as release by eosinophils has been also reported (58). It is tempting to speculate that inhibitory effect of dexamethasone on mediator release may be associated with its inhibitory effect on CD63 mobilization and translocation to the cell periphery. However, further investigation is required to examine the interconnection between inhibitory effects of dexamethasone on mediator release and CD63 mobilization and translocation to the cell periphery.

Various agonists used in our experiments which included IFNγ, C5a/CB and a cocktail of IL-3/IL-5/GM-CSF are known to activate different signal transduction pathways. While JAK2 tyrosine kinase activation appears to be the initiating event for signaling through IL-3/IL-5/GM-CSF (59,60), IFNγ affects eosinophils through its specific receptor and utilizes the Jak/Stat pathway as its long-term (hours) mode of signaling (61,62). C5a-induced stimulation is known to activate heterotrimeric G proteins

(63,64). The association of intracellular translocation and cell surface upregulation of CD63 with activation of these different signal transduction pathways may suggest a central function for CD63 in signal transduction events and, as it has been proposed previously, CD63 may in fact act as a "molecular facilitator" in processes associated with exocytosis.

Members of SNARE fusion complex have been proposed to play a central role in exocytosis (65). Our recent studies have shown that human peripheral blood eosinophils express members of the SNARE fusion complex, including VAMP-2 (66), syntaxin-4 (67) and SNAP-23 (68). In addition to SNAREs, another group of proteins that are proposed to be involved in eosinophil exocytosis are Rho-related family of GTPases. Members of Rho-related GTPases, including Rac-2, have been identified in eosinophils and shown to be critical in the assembly of NADPH oxidase complex prior to the generation of superoxide (69).

The potential interdependence between different elements of eosinophils exocytosis remains the subject of speculation. We are intrigued by the potential interplay between the CD63 and members of SNARE fusion complex and/or Rho-related GTPases and are currently pursuing studies to define the mode of their interaction.

In conclusion, our observations have important implications for expanding current knowledge on the association of tetraspanins with the eosinophil and describe a new potential role for CD63 in the processes leading to eosinophil exocytosis. Our findings provide a new target for future intervention in eosinophil mediator release, which may potentially lead to new therapeutic strategies in the treatment of eosinophil related allergic inflammation and asthma.

References

1. Gleich, G.J., C.R. Adolphson, and K.M. Leiferman. 1993. The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44:85-101.
2. Wardlaw, A.J., R. Moqbel, and A.B. Kay. 1995. Eosinophils: biology and role in disease. *Adv. Immunol.* 60:151-266.
3. Weller, P. 1991. The immunobiology of eosinophils. *N. Engl. J. Med.* 324:1110-1118.
4. Flavahan, N., N. Slifman, G. Gleich, and P. Vanhoutte. 1988. Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscles: role of the epithelium. *Am. Rev. Respir. Dis.* 138:685-688.
5. Hamman, K.J., I. Douglas, and R. Moqbel. 2000. Eosinophil mediators. *In* Asthma and Rhinitis. W.W. Busse, S.T. Holgate, editors. Vol 2. Blackwell Science LTD,Oxford, UK. 394-428.
6. Lacy, P., and R. Moqbel. 2000. Eosinophil cytokines. *Chem. Immunol.* 76:134-155.
7. Dvorak, A.M., and P.F.Weller. 2000. Ultrastructural analysis of human eosinophils. *Chem. Immunol.* 76:1-28.
8. Lacy, P., and R. Moqbel. 1997. Eokines: synthesis, storage and release from human eosinophils. *Mem. Inst. Oswaldo. Cruz.* 92:125-133.
9. Ackerman, S.J., D.A. Loegering, P. Venge, I. Olsson, J.B. Harley, A.S. Fauci, and G.J. Gleich. 1983. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* 131:2977-2982.
10. Lewis, D., J. Lewis, D. Loegering, and G.J. Gleich. 1987. Localization of guinea pig eosinophil major basic protein to the core of the granule. *J. of Cell. Bio.* 77:702-713.

11. Lacy, P., F. Levi-Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J.R. Velazquez, A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon gamma. *Blood.* 91:2508-2516.

12. Lacy, P., S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J.R. Velazquez, S.F. Man, and R. Moqbel. 1999. Rapid mobilization and release of intracellularly stored RANTES in response to interferon-γ in human eosinophils. *Blood.* 94:23-32.

13. Erjefält, J.S., M. Andersson, L. Greiff, M. Korsgren, M. Gizycki, P.K. Jeffery, and G.A. Persson. 1998. Cytolysis and PMD as distinct modes of activation of airway mucosal eosinophils. *J. Allergy Clin. Immunol.* 102:286-294.

14. Moqbel, R., and P. Lacy. 1999. Exocytotic events in eosinophils and mast cells. *Clin. Exp. Allergy.* 29:1017-1022.

15. Capron, M., M. Tomassini, G. Torpier, J.P. Kusnierz, S. MacDonald, and A. Capron. 1998. Selectivity of mediators released by eosinophils. *Int. Arch. Allergy Appl. Immunol.* 88:54-58.

16. Tomassini, M., A. Tsicopoulos, P.C. Tai, V. Gruart, A.B. Tonnel, L. Prin, A. Capron, and M. Capron. 1991. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J. Allergy Clin. Immunol.* 88:365-375.

17. Kobayashi, T., U.M. Vischer, C. Rosnoblet, C. Lebrand, M. Lindsay, R. G. Parton, E. K. Kruithof, and J. Gruenberg. 2000. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Mol. Biol. Cell.* 11:1829-1843.

18. Fukuda, M. 1991. Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem.* 266:21327-21330.

19. Maecker, H.T., S.C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J.* 11:428-442.

20. Skubitz, K.M., K. D. Campbell, J. Iida, and A. P. Skubitz. 1996. CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. *J. Immunol.* 157:3617-3626.

21. Smith, D.A., P.N. Monk, and L.J. Partridge. 1995. Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells. *Mol. Immunol.* 32:1339-1344.

22. Berditchevski, F., and E. Odintsova. 1999. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J. Cell Biol.* 146:477-492.

23. Hallden, G., M. Hadi, H.T. Hong, and G.W. Aponte. 1999. Y receptor-mediated induction of CD63 transcripts, a tetraspanin determined to be necessary for differentiation of the intestinal epithelial cell line, hBRIE 380i cells. *J. Biol. Chem.* 274:27914-27924.

24. Nieuwenhuis, H.K., J.J. van Oosterhout, E. Rozemuller, F. van Iwaarden, and J.J. Sixma. 1987. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood.* 70:838-845.

25. Cham, B.P., J.M. Gerrard, and D.F. Bainton. 1994. Granulophysin is located in the membrane of azurophilic granules in human neutrophils and mobilizes to the plasma membrane following cell stimulation. *Am. J. Pathol.* 144:1369-1380.

26. Knol, E.F., F.P. Mul, H. Jansen, J. Calafat, and D. Roos. 1991. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J. Allergy Clin. Immunol.* 88:328-338.

27. Kitani, S., E. Berenstein, S. Mergenhagen, P. Tempst, and R.P. Siraganian. 1991. A cell surface glycoprotein of rat basophilic leukemia cells close to the high affinity IgE receptor (Fc epsilon RI). Similarity to human melanoma differentiation antigen ME491. *J. Biol. Chem.* 266:1903-1909.

28. Calafat, J., H. Janssen, E.F. Knol, P.F. Weller, and A. 1997. Egesten. Ultrastructural localization of Charcot-Leyden crystal protein in human eosinophils and basophils. *Eur. J. Haematol.* 58:56-66.

29. Egesten, A., J. Calafat, E.F. Knol, H. Janssen, and T.M. Walz. 1996. Subcellular localization of transforming growth factor-alpha in human eosinophil granulocytes. *Blood.* 87:3910-3918.

30. Mahmudi-Azer, S., P. Lacy, B. Bablitz, and R. Moqbel.1998. Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *J Immunol. Methods.* 217:113-119.

31. Moqbel R., J. Barkans, B.L. Bradley, and S.R. Durham, and A.B. Kay. 1992. Application of monoclonal antibodies against major basic protein (BMK-13) and eosinophil cationic protein (EG1 and EG2) for quantifying eosinophils in bronchial biopsies from atopic asthma. *Clin. Exp. Allergy.* 22:265-273.

32. Schleimer R.P. 1993. Glucocorticosteroids: their mechanism of action and use in allergic diseases. In Allergy: principles and practice. E. Middleton, C.E. Reed, E.F.J. Ellis, N.F. Adkinson, J.W. Yunginger, W. Busse, editors. St. Louis, Mosby, 893-925.

33. Jeffery, P.K., R.W. Godfrey, E. Adelroth, F. Nelson, A. Rogers, and S.A. Johansson. 1992. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma: a quantitative light and electron microscopic study. *Am. Rev. Respir. Dis.* 145:890-899.

34. Metzger, W.J., D. Zavala, H.B. Richerson, P. Moseley, P. Iwamota, M. Monick, K. Sjoerdsma, and G.H. Hunninghake. 1987. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs: description of the model and local airway inflammation. *Am. Rev. Respir. Dis.*135:433-440.

35. Djukanovic, R., C.K. Lai, J.W. Wilson, K.M. Britten, S.J. Wilson, W.R. Roche, P.H. Howarth, and S.T. Holgate. 1992. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur. Respir. J.* 5:538-544.

36. Erjefält, J.S., L. Greiff, M. Andersson, E. Matsson, H. Petersen, M. Linden, T. Ansari, P.K. Jeffery, and C.G. Persson. 1999. Allergen-induced eosinophil cytolysis is a primary mechanism for granule protein release in human upper airways. *Am. J. Respir. Crit. Care. Med.*160:304-312.

37. Godthelp, T., A.F.Holm, W.J. Fokkens, P. Doornenbal, P.G. Mulder, E.C. Hoefsmit, A. Kleinjan, E.P. Prens, and E. Rijntjes. 1996. Dynamics of nasal eosinophils in response to a nonnatural allergen challenge in patients with allergic rhinitis and control subjects: a biopsy and brush study. *J. Allergy. Clin. Immunol.* 97:800-811.

38. Erjefält , J.S., and C.G. Persson. 2000. New aspects of degranulation and fates of airway mucosal eosinophils. *Am. J. Respir. Crit. Care. Med.* 61:2074-85.

39. Takasaka, T., A. Kurihara, H. Suzuki, T. Kikushi, K. Hozawa, and H. Wataya. 1990. The differeniation of polyps and their mucosal ultrastrucure. *Am. J. Rhinol.* 4:159-162.

40. Erjefält, J.S., M. Korsgren, M.C. Nilsson, F. Sundler, and C.G.A. Persson. 1997. Association between inflammation and epithelial damage-restitution processes in allergic airways *in vivo. Clin. Exp. Allergy.* 27:1345-1357.

41. Hutson, P.A., M.K. Church, T.P. Clay, P. Miller, and S.T. Holgate. 1988. Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs: I. The association of disordered airway physiology to leukocyte infiltration. *Am. Rev. Respir. Dis.* 137:548-557.

42. Dvorak, A.M., T. Furitsu, L. Letourneau, T. Ishizaka, and S.J. Ackerman. 1991. Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. *Am. J. Pathol.* 138:69-82.

43. Dvorak, A.M., S.J. Ackerman, T. Furitsu, P. Estrella, L. Letourneau, and T. Ishizaka. 1992. Mature eosinophils stimulated to develop in human-cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation. *Am. J. Pathol.* 140:795-807.

44. Mengelers, H.J., T. Maikoe, L. Brinkman, B. Hooibrink, J.W. Lammers, and L. Koenderman.1994. Immunophenotyping of eosinophils recovered from blood and BAL of allergic asthmatics. *Am. J. Respir. Crit. Care. Med.* 149:345-351.

45. Escola, J.M., M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, and H.J. Geuze. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273:20121-20127.

46. Akiyama, T., and H. Ogawara.1991. Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* 201:362-370.

47. Polkowski, K., and A.P. Mazurek. 2000. Biological properties of genistein. A review of in vitro and in vivo data. *Acta. Pol. Pharm.* 57:135-155.

48. Kita H., M. Kato, G.J. Gleich, and R.T. Abraham. 1994. Tyrosine phosphorylation and inositol phosphate production: are early events in human eosinophil activation stimulated by immobilized secretory IgA and IgG? *J. Allergy. Clin. Immunol.* 94:1272-1281.

49. van der Bruggen T., D. Kanters, A.T. Tool, J.A. Raaijmakers, J. W. Lammers, A. J. Verhoeven, L. Koenderman. 1998. Cytokine-induced protein tyrosine phosphorylation is essential for cytokine priming of human eosinophils. *J. Allergy. Clin. Immunol.* 101:103-109.

50. Kato M., H. Kita, K. Tokuyama, and A. Morikawa. 1998. Signal transduction in activation of human eosinophils: G protein-dependent and -independent pathways. *Int. Arch. Allergy. Immunol.* 117:63-67.

51. Coffer P.J., R. C. Schweizer, G.R. Dubois, T. Maikoe, J.W. Lammers, and L. Koenderman. 1998. Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. *Blood.* 91:2547-2557.

52. Ochiai, K., C. Ishihara, and H. Tomioka. 1999. Signal transduction through interferon-gamma receptor on human eosinophils. *Int. Arch. Allergy Immunol.* 118:443-446.

53. Schleimer R.P., and B.S. Bochner BS. 1994. The effects of glucocorticoids on human eosinophils. *J. Allergy. Clin. Immunol.* 94:1202-1213.

54. Wallen N., H. Kita, D. Weiler, and G.J. Gleich. 1991. Glucocorticoids inhibit cytokine-mediated eosinophil survival. *J. Immunol.* 147:3490-3495.

55. Juan M., J. Mullol, J. Roca-Ferrer, M. Fuentes, M. Perez, C. Vilardell, J. Yague, and C. Picado. 1999. Regulation of ICAM-3 and other adhesion molecule expressions on eosinophils in vitro. Effects of dexamethasone. *Allergy.* 54:1293-1298.

56. Adachi T., S. Motojima, A. Hirata, T. Fukuda, N. Kihara, A. Kosaku, H. Ohtake, and S. Makino. 1996. Eosinophil apoptosis caused by theophylline, glucocorticoids, and macrolides after stimulation with IL-5. *J. Allergy. Clin. Immunol.* 98:207-215.

57. Hallam, C., D.I. Pritchard, S. Trigg, and R.P. Eady. 1982. Rat eosinophil-mediated antibody-dependent cellular cytotoxicity: investigations of the mechanisms of target cell lysis and inhibition by glucocorticoids. *Clin. Exp. Immunol.* 48:641-648.

58. Miyamasu, M., Y. Misaki, S. Izumi, T. Takaishi. Y. Morita, H. Nakamura, K. Matsushima, T. Kasahara, and K. Hirai. 1998. Glucocorticoids inhibit chemokine generation by human eosinophils. *J. Allergy Clin. Immunol.* 101:75-83.

59. D'Andrea, R.J., and T.J.Gonda. 2000. A model for assembly and activation of the GM-CSF, IL-3 and IL-5 receptors: insights from activated mutants of the common beta subunit. *Exp. Hematol.* 28:231-243.

60. Woodcock, J.M., C.J. Bagley, and A.F. Lopez. 1999. The functional basis of granulocyte-macrophage colony stimulating factor, interleukin-3 and interleukin-5 receptor activation, basic and clinical implications. *Int. J. Biochem. Cell Biol.* 31:1017-1025.

61. Kotenko, S.V., and S. Pestka. 1999. Jak-Stat signal transduction pathway through the eyes of cytokine class II receptor complexes. Oncogene. 2000;19:2557-2565.

62. Tau G, Rothman P. Biologic functions of the IFN-gamma receptors. *Allergy.* 54:1233-1251.

63. Wetsel, R.A. 1995. Structure, function and cellular expression of complement anaphylatoxin receptors. *Curr. Opin. Immunol.* 7:48-53.

64. Gerard, C., and N.P. Gerard. 1994. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* 12:775-808.

65. Sollner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J.E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature.* 362:318-324.

66. Lacy, P., M. Logan, B. Bablitz, and R. Moqbel. 2001. The fusion protein VAMP-2 is implicated in IFN-γ induced piecemeal degranulation in human eosinophils from atopic individuals. *J. Allergy. Clin. Immunol.* 107, 671-678.

67. Lacy, P., A. Johari, S. Mahmudi-Azer, and R. Moqbel. 2000. Do eosinophils express the exocytotic fusion protein syntaxin-4? *American Journal of Respiratory and Critical Care Medicine.* 161:539a. (abstr.)

68. Lacy, P., A Johri, S. Mahmudi-Azer, and R. Moqbel. 2000. Expression of the SNARE isoform SNAP-23 in human eosinophils. *J. Allergy Clin. Immunol.* 104:S252. (abstr.)

69. Lacy, P., S. Mahmudi-Azer, B. Bablitz, M. Gilchrist, P. Fitzharris, D. Cheng, S.F. Man, G.M. Bokoch, and R. Moqbel. 1999. Expression and translocation of Rac2 in eosinophils during superoxide generation. *Immunology.* 98:244-252.

Chapter 5

General Discussion and Conclusions

A. Immunofluorescence analysis of cytokine and granule protein expression during eosinophil maturation from cord blood-derived CD34$^+$ progenitors

Crystalloid granules are the major storage sites for eosinophil derived mediators. In mature eosinophils a large number of mediators including cationic proteins and cytokines/chemokines have been shown to be stored in these unique structures (1,2). Although mediator expression and their subsequent storage in crystalloid granules appears to be central to the proinflammatory function of eosinophils, these areas of eosinophil studies remain relatively unknown. In this study, I examined the morphological characteristics of eosinophilopoiesis and the processes leading to granulogenesis. I studied the expression and pattern of granule storage of a cytokine (IL-6) and a chemokine (RANTES) in parallel with cationic proteins in maturing eosinophils. Previous studies have only partially examined the morphological characteristics of eosinophilopoiesis and the formation of crystalloid granule using electron microscopy and *in vitro* culture systems (3,4). However, none of these studies examined the expression and pattern of granule storage of cytokines in parallel with cationic proteins in maturing eosinophils.

Using double immunofluorescent staining together with CLSM, we examined the evolution of the expression pattern of cytokines/chemokines and granule cationic proteins during differentiation and maturation of CD34$^+$ progenitor-derived eosinophils in an *in vitro* culture system. Our observations suggested that MBP, ECP, IL-6 and RANTES are not expressed at detectable levels in freshly purified CD34$^+$ cells. However, maturing eosinophils exhibited positive immunostaining for all of these proteins between days 16 and 28 of culture. During early stages of eosinophil culture from CD34$^+$progenitors (day

16), immunoreactivity against these mediators localized to granular structures and discrete immunostaining was observed around the periphery but not in the centre of these granular structures. By day 28 of culture, eosinophil-like cells showed evidence for the acquisition of crystalloid granule-like morphology analogous to that observed in mature peripheral blood eosinophils.

The early expression of mediators and their storage in granular structures is an important finding, since it indicates that maturing eosinophils may have the capacity to exhibit local immune-effector and immune-regulatory function during differentiation and maturation in the bone marrow as well as at local (*in situ*) tissues. In fact, previous studies have shown that maturing eosinophils generated from bone marrow-derived CD34$^+$ cells are capable of mediator release at early stages of their development (5). Whether maturing eosinophils, known to express and store these mediators, play a role in immune effector and immune regulatory processes in the bone marrow or the local tissue, is an important issue that requires further investigation.

Interestingly, in day 16 of culture, maturing eosinophils appeared to contain a large number of small granular structures. Later during culture (day 19) this population of small granules appeared to be replaced by a smaller number of larger granules. The decrease in number and increase in size of the granular structures in day 19 could potentially be the outcome of one of the following two processes:

1) A proportion of smaller size granules may be released to extracellular space due to sub- or supra-optimal concentrations of growth factors and cytokines in culture media, with the remaining granules possibly increasing in size by incorporating mediator containing vesicles. These presumably shuttle mediators between maturing granules and

Golgi compartment. The perceived increase in the size of the granules may also have been caused by mediator synthesis within the granule itself. Indeed, a recent study by Dvorak et al. has shown that RNA as well as ribosomes are detectable in granules of mature human mast cells. In this study, the authors have suggested that aspects of mediator synthesis processes may occur within granules (6). It is tempting to speculate that, as in the granules of mature human mast cells, granules of maturing eosinophils may contain protein synthesis machinery which they can use to synthesize prestored mediators. This process may be at least partially independent of the Golgi compartment.

2) The increase in the size and decrease in the number of granular structures may have been caused by intracellular fusion of smaller granules resulting in the formation of larger granules. In a previous study researchers, using whole-cell patch-clamp capacitance measurements, showed that homotypic fusion of unit granules takes place in maturing eosinophil cultures, *in vitro* (5). In this study, the authors have proposed that mature large specific granules are formed by homotypic fusion of unit granules of similar size (5).

The intracellular fusion of granules has been also described in mature human eosinophils in a process known as compound exocytosis. Here a number of crystalloid granules merge intracellularly to form a large intracellular membrane-bound pool of mediators, before release to the extracellular space (7,8). Merging of mature crystalloid granules of eosinophils in compound exocytosis suggests the presence of the putative elements necessary for granule membranes to fuse together. Similar mechanisms may underlie the merging of smaller granules in the earlier days of granulogenesis in semi-solid cultures leading to the formation of larger granular structures by day 19. Nevertheless, further studies are needed to confirm or disprove the above speculations.

Another interesting aspect of granule maturation is the differential localization and compartmentalization of mediators within the granules at various days of culture. During the early stages of culture, immunoreactivity against MBP, ECP, IL-6 and RANTES appeared to be localized to the periphery but not to the centre of these granule-like structures. Nonetheless, by day 28, some of the granular structures showed patterns of immunoreactivity similar to that of mature crystalloid granules. While MBP immunoreactivity was observed to be localized to the core compartment, immunoreactivity against other mediators such as ECP, IL-6, and RANTES localized to the matrix compartment. Such differential localization and compartmentalization of mediators may indicate the potential presence of as yet unidentified sorting molecules and mechanisms in maturing eosinophil granules. Mediator trafficking and sorting within the ER and Golgi compartments have been the subject of intense investigations. However, mediator trafficking and differential localization within secretory granules remain generally unknown.

Taken together, the intragranular processes associated with granule structure and size as well as mediator compartmentalization within granule structures are intriguing and require further studies. It was due to the lack of appropriate antibody markers and limited cell numbers in our *in vitro* culture system that we were unable to fully investigate the molecules and mechanisms underlying the changes regulating granular size, structure and differential compartmentalization of mediators within both core and matrix. Such studies require a larger number of cells, which may be achieved in future by using more advanced *in vitro* culture systems.

B. Selective mediator release in eosinophils

The activation of eosinophils and the release of eosinophil granule mediators in airway mucosa is considered a critical process in the pathogenesis of asthma, allergic rhinitis, and nasal polyposis (10-14). There is also increasing evidence for the presence of cationic granule products, such as MBP, ECP in airway tissues and lavae (15-17). However, despite intensive studies on airway eosinophils, little is known about the molecules and mechanisms regulating degranulation of these cells in the inflamed airway tissue.

Piecemeal degranulation or selective mediator release, whereby the granular content is fully or partially released from intracellular granules, is a prominent mechanism by which eosinophils discharge extracellularly their proinflammatory mediators (18,19). There is general agreement that PMD is a true phenomenon, but the regulatory molecular mechanisms associated with this event in airway mucosa remain largely unexplored.

Having studied mediator expression, storage, and compartmentalization in maturing eosinophils we investigated the mobilization and release of prestored mediators in crystalloid granules of mature eosinophils following agonist stimulation. In our studies on eosinophil exocytosis, we used RANTES, a chemokine prestored in mature peripheral blood eosinophils, as a marker for selective mediator mobilization and release.

Indeed we showed that RANTES immunoreactivity in human eosinophils was associated with electron lucent matrix of the crystalloid granules. This was based on its close apposition to the crystalline core protein marker, MBP. Our data also indicated that IFNγ stimulation of eosinophils rapidly (10 min) activated the selective release of RANTES from intracellular pools. Prior to its release, RANTES immunoreactivity was observed to be selectively mobilized and appeared to localize to a population of granules adjacent to the cell periphery, while MBP immunoreactivity remained relatively unchanged.

Using subcellular fractionation on resting eosinophils we were able to detect RANTES immunoreactivity in two distinct compartments. Interestingly, RANTES was detected in a readily releasable pool distinct from MBP- and EPO-containing granules. This rapidly mobilizable pool of RANTES was associated with light membrane fractions and was released to extracellular space 10 min after IFNγ stimulation. Following 60 min of IFNγ stimulation light-membrane associated RANTES immunoreactivity was restored while crystalloid granule-associated RANTES was equally reduced. This suggests that RANTES may have translocated from crystalloid granule-associated pool to a light membrane-associated pool. Indeed, crystalloid granule associated RANTES may have acted as a reservoir for the readily releasable pool.

The presence of a similar readily releasable pool of mediators has been detected in pancreatic endocrine β-cells. Measuring cell capacitance, insulin exocytosis, provoked by a modest elevation of Ca^{2+}, shown to be resolved into a rapid release phase followed by a slower but sustained release phase (18). The former is thought to represent rapid exocytosis of a fusion competent pool of granules (Readily Releasable Pool, RRP) while the latter likely represents delayed exocytosis of granules that have to be recruited into the RRP from reserve pools (19). Interestingly, exocytosis of this readily releasable pool of granules is independent of ATP and Ca $^{2+}$, indicating that they have progressed beyond ATP-dependent priming. In contrast, the slower recruitment phase was both Ca $^{2+}$ and ATP-dependent. This suggests that it represents delayed exocytosis of granules that have to be recruited into the RRP from reserve pools and require maturation to achieve fusion-competency (19).

Our observations suggest that eosinophils possess a similar mechanism for selective piecemeal release of mediators prestored in either small secretory vesicles or crystalloid granules. We propose that the rapid release of RANTES in activated eosinophils may be associated with a primed population of small secretory vesicles. As well the further budding of small secretory vesicles from crystalloid granules may account for the putative restoration of this population of secretory vesicles. Indeed, such small secretory vesicles may be responsible for shuttling proteins between crystalloid granules and plasma membrane. Selective release of eosinophil granule proteins and its association with vesicle budding from crystalloid granules has been described in earlier reports (20). A similar pattern of piecemeal degranulation has also been proposed based on electron microscopy sections of degranulating eosinophils, *in vivo* (20).

Little is known about underlying molecular mechanisms and signal transduction pathways associated with selective mediator sorting, mobilization and piecemeal degranulation. The gradual, time-dependent and selective mobilization of RANTES immunoreactivity to granules adjacent to the cell periphery may indicate the presence of a specific mechanism regulating intragranular mediator sorting and translocation. It is possible that upon selective sorting and recruitment of mediators into small secretory vesicles, the latter bud from a distal population of granules and merge with granules proximal to plasma membrane in a stepwise fashion.

To date, three basic classes of vesicles associated with intracellular trafficking of proteins, have been identified: COPII, COPI and clathrin coated vesicles (21). While COPII-coated vesicles have been proposed to facilitate cargo translocation in ER (22), COPI-coated vesicles are thought to facilitate mediator translocation in the Golgi compartment. Indeed COPI has been proposed to be widely associated with membrane budding and fusion events in the Golgi compartment (23). Clathrin-coated vesicles transport lipids and proteins between intracellular membrane compartments. Clathrin-coated vesicles are the most prominent of the carriers in the endocytic pathway, (between the plasma membrane and early endosomes), and in the secretory pathway, (between the trans-Golgi network and late endosomes) (24). Clathrin coats act at the plasma membrane to form endocytic transport vesicles, at the trans-Golgi network (TGN) to form endosome-targeted vesicles, as well as possibly at other transport sites. It is tempting to speculate that clathrin coats may be responsible for the budding of small secretory vesicles from eosinophil crystalloid granules and subsequent translocation of RANTES from a reservoir pool to a light membrane-associated pool.

One fascinating aspect of the process of mediator mobilization and release in eosinophils is its selectivity. While a large number of mediators are prestored in crystalloid granules, mediators are selectively mobilized and released. Two particular family members, p23 and p24, which are especially abundant in Golgi membrane, are proposed to be associated with selective mediator translocation in Golgi compartment (25). Both p23 and p24 are highly concentrated in Golgi-derived COPI-coated vesicles

(26). It has been proposed that the cytoplasmic tails of the p24 family of proteins act as a coat receptor and are important for coat assembly, while their luminal domains have been proposed to bind cargo on the opposite side of the membrane (25-27). A similar process may facilitate the selective cargo recruitment to small secretory vesicles and orchestrate the piecemeal degranulation in eosinophils. Yet, the expression of clathrin, COPI, COPII, p24 and p23 and their potential association with selective mediator mobilization and release in eosinophils remain unknown and perhaps should be included in future studies.

C. The association of the tetraspanin, CD63, with eosinophil selective mediator release

As transmembrane proteins with extracellular domains, tetraspanins have been proposed to act as "adaptors" or "molecular facilitators". By adapting and facilitating the formation of functional signaling complexes and increasing their stability, tetraspanins are thought to coordinate various cellular functions such as cell proliferation, differentiation, activation and adhesion (28).

The tetraspanin, CD63, is a protein of single long open reading frame of 238 amino acids containing four putative transmembrane regions and three N-glycosylation sites (29). CD63 is identical to ME491, an antigen reported as a melanoma-associated antigen. Although, CD63 is not related to the lysosomal glycoprotein family, it contains the putative lysosomal targeting signal Gly-Tyr in its short cytoplasmic tail (29).

A number of studies have examined the CD63 expression and potential function in various cell types, including neutrophils, basophils, platelets, and endothelial cells (30-36). Yet, its expression and function in human eosinophils remain widely unexplored.

To further our understanding on the selective mobilization and release of prestored RANTES in agonist-activated eosinophils, we examined the participation of CD63 in exocytosis. In resting peripheral blood eosinophils CD63 localized to the membrane and/or matrix compartment of all MBP $^+$ granules. Following IFNγ (10 min) or C5a/CB stimulation of eosinophils, intracellular CD63 immunoreactivity appeared to be confined to membranes of those crystalloid granules adjacent to the plasma membrane. Indeed, intracellular mobilization and translocation of CD63 to the cell periphery upon IFNγ stimulation coincided with that of RANTES. We also observed that upon mediator release, CD63 expression was upregulated on plasma membrane of stimulated cells. Interestingly, peripheral blood eosinophils of asthmatics showed higher CD63 expression on their surface membrane when compared with eosinophils of non-asthmatic individuals (Moqbel, Mahmudi-Azer, unpublished data).

As discussed above, our subcellular fractionation studies of IFNγ-activated eosinophils, revealed that RANTES immunoreactivity localized to two distinct pools: 1) a readily releasable light membrane-associated pool of small mediator-containing vesicles which released their content to extracellular space within minutes after stimulation, and 2) crystalloid granule-associated pool which may act as a reservoir for the light membrane-associated pool. It is thought that budding of mediator-containing vesicles may selectively and gradually evacuate the content of crystalloid granules (37-39). Our studies on CD63 localization in activated eosinophils indicated a convergence between CD63 and mediator mobilization and release. As a consequence, we propose that immunoreactivity against RANTES together with that of CD63, (both of which are originally associated with MBP^+ granules), translocate to cell periphery in order to restore the light membrane-associated pool of RANTES (Fig. 1).

Vesicle budding in other intracellular compartments involves a number of different molecules. In *trans*-Golgi network (TGN) the vesicle formation is associated with selective capture of the cargo molecules at the budding site. This occurs together with the recruitment of regulatory and structural proteins required for local deformation and fission of the membrane pit and for delivery and fusion of the resulting vesicle to the target membrane (40). Clathrin-based systems are responsible for a large fraction of the vesicular traffic originating from TGN. During vesiculation, cytosolic clathrin assemble into a coat that drives membrane deformation. Clathrin is also associated with a number of other proteins, some of which are directly involved in the recruitment of cargo. They are referred to as adaptors because they link clathrin coats and cargo sorting (41).

Adaptor proteins constitute a large family of monomeric and heterotetrameric complexes, which are found to varying extents in all nucleated cells from yeast to humans. The founding members are the mammalian AP-1 complex, specific for traffic from the TGN to the endosome, and AP-2 adaptor complexes specific for traffic originating at the plasma membrane and the endosome (42,43). A second mammalian AP-1 complex, specific for the endosomal compartment, has been also identified (44,45). The fifth family member, AP-3, is mostly associated with endosomal membranes (46,47). To my knowledge, there are no studies addressing the identification of the expression of various members of the AP family in eosinophils or to examine the potential association of CD63 with members of this family. The presence of a putative

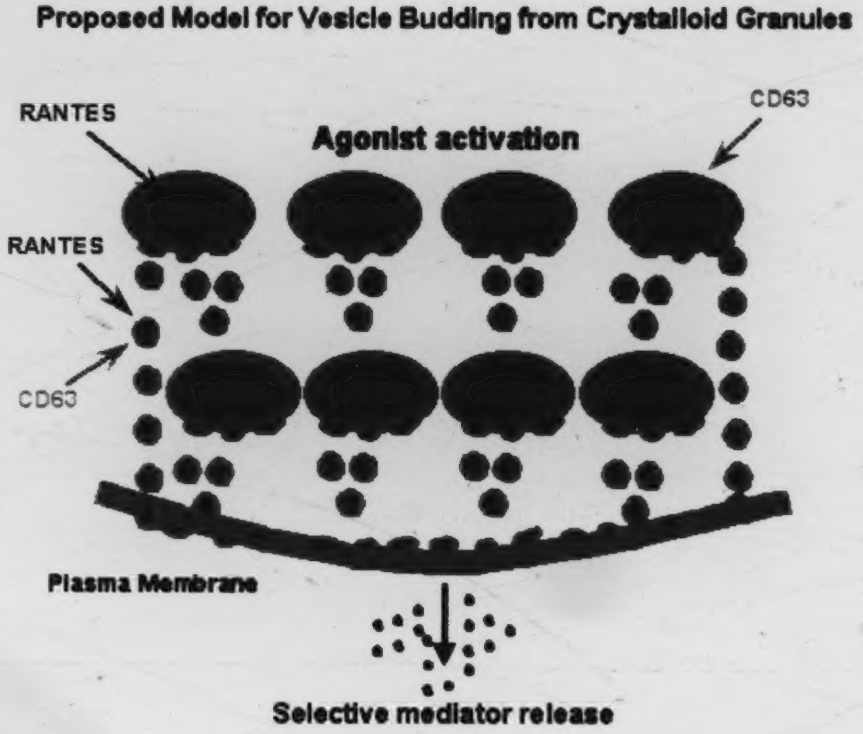

Proposed Model for Vesicle Budding from Crystalloid Granules
RANTES
Agonist activation
CD63
RANTES
CD63
Plasma Membrane
Selective mediator release

Fig. 5.1: Proposed model for vesicle budding from crystalloid granules

lysosomal targeting signal on CD63 (29) and its association with PI4 kinases (48,49), tyrosin kinases such as Lyn and Hck (50), and selective mediator mobilization and release renders this molecule a suitable candidate for potential association with members of the AP family.

The association of CD63 with various signal transduction pathways highlights a potential central role for this molecule in cell activation and mediator release. Various agonists used in our experiments, which included IFNγ, C5a/CB and a cocktail of IL-3/IL-5/GM-CSF are known to activate distinct signal transduction pathways. While JAK2 tyrosine kinase activation appears to be the initiating event for signaling through IL-3/IL-5/GM-CSF (51,52), IFNγ activates eosinophils through its specific receptor and utilizes the JAK-2/STAT-1 pathway as its long-term (hours) mode of signaling (53,54). C5a-induced stimulation is known to activate heterotrimeric G proteins (55,56). The association of intracellular translocation and cell surface upregulation of CD63 with activation of these different signal transduction pathways may suggest a facilitating role for CD63 in signal transduction events leading to exocytosis. These may be a novel role for a member of the tetraspanin family of proteins.

Members of SNARE fusion complex have been proposed to play a central role in exocytosis (57). SNARE proteins have been proposed to mediate all intracellular membrane fusion events. There are over 30 SNARE family members in mammalian cells and each is found in a distinct subcellular compartment. It is likely that SNAREs encode aspects of membrane transport specificity but the mechanism by which this specificity is achieved remains controversial (58). Our recent studies have shown that human peripheral blood eosinophils express non-neuronal members of the SNARE fusion complex, including VAMP-2 (59)(vesicular v-SNARE), syntaxin-4 (60) and SNAP-23 (61) (target t-SNAREs). In a recent study on human neutrophil exocytosis, syntaxin 6 was shown to localize mainly to the plasma membrane of human resting neutrophils, whereas SNAP-23 was shown to localize primarily in the mobilizable tertiary and specific granules. Interestingly, in this study, the authors showed that antibodies against SNAP-23, introduced into electropermeabilized neutrophils, inhibited Ca^{2+} and GTPγS induced exocytosis of CD67-enriched specific granules, while negligibly affecting exocytosis of the CD63-enriched azurophilic granules. These data may indicate that distinct molecular processes may regulate mediator release from CD67-enriched specific granules and CD63-enriched azurophilic granules (62).

In addition to SNAREs, another group of proteins that are proposed to be involved in eosinophil exocytosis are the Rho-related family of GTPases. In fact, members of Rho-related GTPases, including Rac-2, have been identified in eosinophils and shown to be critical in the assembly of NADPH oxidase complex prior to the superoxide generation (63). The potential interdependence between different elements of eosinophils exocytosis remains the subject of speculation. We are intrigued by the potential interplay between the CD63, members of SNARE fusion complex and/or Rho-related GTPases and our laboratory is vigorously currently pursuing studies to define the mode and pathways of such potential interactions.

In conclusion, much remains to be known about complicated molecular mechanisms underlying eosinophil mediator release. It is, however, clear that selective and local inhibition of eosinophil mediator release are strategies worth pursuing which may potentially lead to novel therapeutic approaches in the treatment of allergic inflammatory diseases. Our observations on eosinophil granulogenesis, mediator expression/storage and compartmentalization release have important implications for expanding current knowledge on processes underlying eosinophil maturation. Furthermore, our studies on selective mediator release and the close association of CD63 with cell activation and piecemeal degranulation of eosinophils may open new horizons in existing knowledge into the biological functions of tetraspanins. Taken together, the data presented in this thesis have initiated a number of challenging and exiting questions. Chief among them is the precise role of CD63 in signal transduction pathways and its association with membrane trafficking during eosinophil activation. I also hope that these findings will provide conceptual bases for the development of new therapeutic strategies toward the goal of modulating cell activation and mediator release in eosinophil-related allergic inflammation, specially asthma.

References

1. Lacy, P., and R. Moqbel. 2000. Eosinophil cytokines. *Chem. Immunol.* 76, 134-155.
2. Ackerman, S.J., D.A. Loegering, P. Venge, I. Olsson, J.B. Harley, A.S. Fauci, and G.J. Gleich. 1983. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* 131, 2977-2982.
3. Scott, R.E., and R.G. Horn. 1970. Fine structural features of eosinophilic granulocyte development in human bone marrow. *J. Ultrastruct. Res.* 33, 16-28.
4. Popken-Harris, P., J. Checkel, D. Loegering, B. Madden, M. Springett, G. Kephart, and G.J. Gleich. 1998. Regulation and processing of a precursor form of eosinophil granule major basic protein (ProMBP) in differentiating eosinophils. *Blood.* 92, 623-631.
5. Scepek, S., and M. Lindau. 1997. Exocytotic competence and intergranular fusion in cord-blood-derived eosinophils during differentiation. *Blood.* 89, 510-518.
6. Dvorak, A.M., E.S. Morgan, L.M. Lichtenstein, P.F. Weller, and R.P. Schleimer. 2000. RNA is closely associated with human mast cell secretory granules, suggesting a role(s) for granules in synthetic processes. *J. Histochem. Cytochem.* 48, 1-12.
7. Scepek, S., and M. Lindau. 1993. Focal exocytosis by eosinophils-compound exocytosis and cumulative fusion. *EMBO. J.* 12,1811-1817.

8. Newman, T.M., M. Tian, and B.D. Gomperts. 1996. Ultrastructural characterization of tannic acid-arrested degranulation of permeabilized guinea pig eosinophils stimulated with GTP-gamma-S. *Eur. J. Cell Biol.* 70, 209-220.

9. Frigas E., and G.J. Gleich. 1986. The eosinophil and the pathophysiology of asthma. *J. Allergy Clin. Immunol.* 77,527-537.

10. Bousquet J., P. Chanez, J.Y. Lacoste, G. Barneon, N. Ghavanian, I. Enander, P. Venge, S. Ahlstedt, J. Simony-Lafontaine, and P. Godard. 1990. Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 323,1033-1039.

11. Busse, W.W., J.B. Sedgwick, N.N. Jarjour, and W.F. Calhoun. 1994. Eosinophils and basophils in allergic airway inflammation. *J. Allergy Clin. Immunol.* 94,1250-1254.

12. Azzawi, M., B. Bradley, P.K. Jeffery, A.J. Frew, A.J. Wardlaw, and G. Knowles. 1990. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis.* 142,1407-1413.

13. Filley, W.V., K.E. Holley, G.M. Kephart, and G.J. Gleich. 1982. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* 2,11-16.

14. Virchow, J.C. J.r., U. Holscher, and C. Virchow. 1992. Sputum ECP levels correlate with parameters of airflow obstruction. *Am. Rev. Respir. Dis.* 146, 604-606.

15. Klementsson, H., C. Svensson, M. Anderson, P. Venge, U. Pipkorn, and C.G.A.Persson. 1991. Eosinophils, secretory responsiveness, and glucocorticoid-

induced effects on the allergic nasal mucosa during a weak pollen season. *Clin. Exp. Allergy* 21,705-710.

16. Dvorak, A.M., T. Furitso, L. Letourneau, T. Ishizaka, and L. Ackerman. 1997. Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures with human recombinant interleukin-5. I. Piecemeal degranulation of specific granules and distribution of Chacot-Leiden crystal protein. *Am. J. Pathol.* 138,69-82.
17. Weller, P.F., and A.M. Dvorak. 1994. Human eosinophils: development, maturation, and functional morphology. *In:* Busse WW, Holgate ST, editors. Asthma and rhinitis. Boston: Blackwell; p. 255-74.
18. Erjefalt, J.S., M. Andersson, L. Greiff, M. Korsgren, M. Gizycki, P.K. Jeffery, and G.A. Persson. 1998. Cytolysis and piecemeal degranulation as distinct modes of activation of airway mucosal eosinophils. *J. Allergy Clin. Immunol.* 102, 286-294.
19. Eliasson, L,, E. Renström, W.G. Ding, P. Proks, P.Rorsman. 1997. Rapid ATP-dependent priming of secretory granules precedes Ca $^{2+}$-induced exocytosis in mouse pancreatic B-cells. *J. Physiol.* 503,399–412.
20. Dvorak, A. 1994. Ultrastructural studies on mechanisms of human eosinophil activation and secretion. *In* Eosinophils in Allergy and Inflammation. G.J. Gleich, and A.B. Kay eds. New York, Marcel Dekker, Inc. p159.
21. Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66,511-548.

22. Wieland, F., and C. Harter. 1999. Mechanisms of vesicle formation: insights from the COP system. *Curr. Opin. Cell Biol.* 11,440-446.

23. Lowe, M., and T.E. Kreis. 1998. Regulation of membrane traffic in animal cells by COPI. *Biochim. Biophys. Acta.* 14, 53-66.

24. Kirchhausen, T., J.S. Bonifacino, and H. Riezman. 1997. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. *Curr. Opin. Cell Biol.* 9, 488–495.

25. Rothman, J.E., and F.T. Wieland. 1996. Protein sorting by transport vesicles. *Science* 272, 227–234.

26. Schimmöller, F.B., B. Singer-Kruger, S. Schröder, U. Krüger, C. Barlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO. J.* 14, 1329–1339.

27. Nickel, W., B. Brugger, and F.T. Wieland. 1998. Protein and lipid sorting between the endoplasmic reticulum and the Golgi complex. *Semin. Cell. Dev. Biol.* 9:493-501.

28. Maecker, H.T., S.C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J.* 11:428-442.

29. Metzelaar, M.J., P.L. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, and H.C. Clevers. 1991. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* 266, 3239-3245.

30. Skubitz, K.M., K. D. Campbell, J. Iida, and A. P. Skubitz. 1996. CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. *J. Immunol.* 157:3617-3626.

31. Smith, D.A., P.N. Monk, and L.J. Partridge. 1995. Antibodies against human CD63 activate transfected rat basophilic leukemia (RBL-2H3) cells. *Mol. Immunol.* 32:1339-1344.

32. Berditchevski, F., and E. Odintsova. 1999. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J. Cell Biol.* 146:477-492.

33. Hallden, G., M. Hadi, H.T. Hong, and G.W. Aponte. 1999. Y receptor-mediated induction of CD63 transcripts, a tetraspanin determined to be necessary for differentiation of the intestinal epithelial cell line, hBRIE 380i cells. *J. Biol. Chem.* 274:27914-27924.

34. Cham, B.P., J.M. Gerrard, and D.F. Bainton. 1994. Granulophysin is located in the membrane of azurophilic granules in human neutrophils and mobilizes to the plasma membrane following cell stimulation. *Am. J. Pathol.* 144:1369-1380.

35. Knol, E.F., F.P. Mul, H. Jansen, J. Calafat, and D. Roos. 1991. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J. Allergy Clin. Immunol.* 88:328-338.

36. Kitani, S., E. Berenstein, S. Mergenhagen, P. Tempst, and R.P. Siraganian. 1991. A cell surface glycoprotein of rat basophilic leukemia cells close to the high affinity IgE receptor (Fc epsilon RI). Similarity to human melanoma differentiation antigen ME491. *J. Biol. Chem.* 266:1903-1909.

37. Lacy, P., S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J.R. Velazquez, S.F. Man, and R. Moqbel. 1999. Rapid mobilization and release of intracellularly stored RANTES in response to interferon-γ in human eosinophils. *Blood.* 94:23-32.

38. Dvorak, A.M., T. Furitsu, L. Letourneau, T. Ishizaka, and S.J. Ackerman. 1991. Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. *Am. J. Pathol.* 138:69-82.

39. Dvorak, A.M., S.J. Ackerman, T. Furitsu, P. Estrella, L. Letourneau, and T. Ishizaka. 1992. Mature eosinophils stimulated to develop in human-cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation. *Am. J. Pathol.* 140:795-807.

40. Kirchhausen, T. 1999. Adaptors for clathrin-mediated traffic. *Annu. Rev. Cell. Dev. Biol.* 15, 705-732.

41. Mallet, W.G., and F.M. Brodsky. 1996. A membrane-associated protein complex with selective binding to the clathrin coat adaptor AP-1. *J. Cell Sci.* 109, 3059–3068.

42. Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO. J.* 7, 919-929.

43. Robinson, M.S., and B.M. Pearse. 1986. Immunofluorescent localization of 100 K coated vesicle proteins. *J. Cell Biol.* 102,48-54.

44. Lewin, D.A., and I. Mellman. 1998. Sorting out adaptors. *Biochim. Biophys. Acta* 1401, 129-45.

45. Takatsu, H., M. Sakurai, H.W. Shin, K. Murakami, and K. Nakayama. 1998. Identification and characterization of novel clathrin adaptor-related proteins. *J. Biol. Chem.* 273, 24693-700.

46. Simpson, F., A.A. Peden, L. Christopoulou, M.S. Robinson. 1997. Characterization of the adaptor-related protein complex, AP-3. *J. Cell Biol.* 137,835-45.

47. Dell'Angelica, E.C., H. Ohno, C.E. Ooi, E. Rabinovich, K.W. Roche, and J.S. Bonifacino. 1997. AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO. J.* 16, 917-28.

48. Yauch, R.L., and M.E. Hemler. 2000. Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem. J.* 2000 Nov 1;351 Pt 3:629-37.

49. Berditchevski, F., K.F. Tolias, K. Wong, C.L. Carpenter, and M.E. Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* 31, 2595-2598.

50. Skubitz, K.M., K. D. Campbell, J. Iida, and A. P. Skubitz. 1996. CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. *J. Immunol.* 157:3617-3626.

51. D'Andrea, R.J., and T.J.Gonda. 2000. A model for assembly and activation of the GM-CSF, IL-3 and IL-5 receptors: insights from activated mutants of the common beta subunit. *Exp. Hematol.* 28:231-243.

52. Woodcock, J.M., C.J. Bagley, and A.F. Lopez. 1999. The functional basis of granulocyte-macrophage colony stimulating factor, interleukin-3 and interleukin-5 receptor activation, basic and clinical implications. *Int. J. Biochem. Cell Biol.* 31:1017-1025.

53. Kotenko, S.V., and S. Pestka. 1999. Jak-Stat signal transduction pathway through the eyes of cytokine class II receptor complexes. Oncogene. 2000;19:2557-2565.

54. Tau G, Rothman P. Biologic functions of the IFN-gamma receptors. *Allergy.* 54:1233-1251.

55. Wetsel, R.A. 1995. Structure, function and cellular expression of complement anaphylatoxin receptors. *Curr. Opin. Immunol.* 7:48-53.

56. Gerard, C., and N.P. Gerard. 1994. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* 12:775-808.

57. Sollner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J.E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature.* 362:318-324.

58. Chen, Y.A., and R.H. Scheller. 2001. SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell Biol.* 2, 98-106.

59. Lacy, P., M. Logan, B. Bablitz, and R. Moqbel. 2000. The fusion protein VAMP-2 is implicated in IFN-γ induced piecemeal degranulation in human eosinophils from atopic individuals. (Submitted).

60. Lacy, P., A. Johari, S. Mahmudi-Azer, and R. Moqbel. 2000. Do eosinophils express the exocytotic fusion protein syntaxin-4? *American Journal of Respiratory and Critical Care Medicine.* 161:539a. (abstr.)

61. Lacy, P., A Johri, S. Mahmudi-Azer, and R. Moqbel. 2000. Expression of the SNARE isoform SNAP-23 in human eosinophils. *J. Allergy Clin. Immunol.* 104:S252. (abstr.)

62. Martin-Martin, B., S.M. Nabokina, J. Blasi, P.A. Lazo, and F. Mollinedo. 2000. Involvement of SNAP-23 and syntaxin 6 in human neutrophil exocytosis. *Blood* 96, 2574-2583.

63. Lacy, P., S. Mahmudi-Azer, B. Bablitz, M. Gilchrist, P. Fitzharris, D. Cheng, S.F. Man, G.M. Bokoch, and R. Moqbel.1999. Expression and translocation of Rac2 in eosinophils during superoxide generation. *Immunology.* 98:244-252.

Appendix I

Confocal Laser Scanning Microscopy and Immunofluorescent Staining Techniques in Detection and Localization of Mediators in Eosinophils[1]

1.[1] Author: S. Mahmudi-Azer. Portions of this chapter were published in *Human Airway Inflammation: Sampling Techniques an Analytical Protocols,* D.F. Rogers and L.E. Donelly (Editors), Human Press Inc. I wrote the section on confocal laser scanning microscopy.

Introduction

Tracing the movement of various mediators inside the cell and their localization to different subcellular components are some of the fascinating aspects of the modern cell and molecular biology. Advanced methods of confocal laser scanning microscopy, combined with immunofluorescent staining, can facilitate the detection and localization of mediators such as cytokines and chemokines to different intracellular compartments. These methods can also be used to trace the intracellular movement of mediators.

Conventional light microscopy

Microscopy has been a mainstay of biology, ever since Leeuwenhock, (1689-1723) used a simple microscope with a magnification of 30-200 times and a resolution of approximately 1 μm. It was many years later that the addition of a second lens led to the evolution of the compound microscope, which has contributed fundamentally to the development of various aspects of biological sciences. In 1876, Ernst Abbe established the foundation of light microscopy and hypothesized that the theoretical maximum resolution of the light microscope (0.2 μm) was limited by the wavelength of light. Since then, the major focus in light microscopy has been on developing methods of illumination and promoting contrast (1).

In conventional light microscopy, "out of focus information" (which will be further discussed) has been a major obstacle, since it contributes to lessened visibility and loss of distinguishable structures (2). One solution to the limitations of light microscopy was the introduction and use of electron microscopy in which electrons are employed to form the image. Since electrons have a shorter wavelength (0.2 ηm) than light, they produce better resolution of the ultrastructural details. However, the complex preparation procedures are lengthy and can be damaging to the specimen. This technique also suffers from fixation and sectioning artifacts. In addition, the electron microscope provides only two-dimensional images, which are difficult to reconstruct (3).

Confocal laser scanning microscopy

After several decades of effort, the invention of laser as a source of light led to the development of confocal laser scanning microscopy CLSM (originally introduced by Maiman in 1960). Since laser can produce light beams with a very high degree of monochromaticity and polarization, the use of laser as a light source overcame various difficulties in conventional light and electron microscopy. An appropriate laser could serve as a valuable light source in those modes of microscopy where monochromaticity, high intensity and a high degree of coherence and polarization are important (4,5).

Confocal microscopy offers several advantages over conventional light and electron microscopy. In conventional light microscopy, in addition to the plane in which the objective lens is focused, information is collected from much of the depth or volume of the specimen and the collected information is uniformly and simultaneously illuminated. This leads to out-of-focus blur from areas above and below the focal plane of interest. Out-of-focus light reduces contrast and decreases resolution, making it difficult to distinguish various cellular structures. Whereas, in confocal microscopy the shallow depth of field (0.5-1.5 μm) allows information to be collected from a well-defined optical section. Furthermore, in confocal microscopy the illumination is not simultaneous, but sequential and is focused as a spot on one volume element of the specimen at a time (6,7). Consequently the "out-of-focus information" is virtually eliminated, which results in an increase in contrast, clarity, and detection sensitivity. Depending on the specific microscope design, wavelength of light, the quality of objective lens, and confocal microscope setting, the spot size may be as small as 0.25 μm in diameter and 0.5 μm in depth (8,9).

In addition to the virtual elimination of "out-of-focus information", the confocal microscope optically scans the specimen so that the physical sectioning artifact observed with light and electron microscopes are eliminated. As optical sectioning is noninvasive, living, as well as fixed, cells can be observed with greater clarity. Moreover, the specimen can be optically sectioned not only in the xy plane (perpendicular to the optical axis of the microscope), but also vertically (parallel to the optical axis of the microscope) in the xz or yz plane (10,11).

Confocal imaging systems are based on the principle of Minsky (12), which states that both the illumination and detection (imaging) systems are focused on the same single volume element of the specimen. The illumination volume elements are sampled in such a way that a spatial filter removes most of the signals from outside the plane of focus and further reduces out-of-focus information. Thus, the illumination, specimen, and detector all have a single focus, that is, they are confocal (13).

It is now agreed that confocal microscopy not only bridges the gap between light and electron microscopy, but provides a means to observe structural components of living cells and tissues in three dimensions without fixation or physically sectioning artifacts.

To achieve confocal imaging, excitation light (from a laser source) is directed toward the specimen. The beams of light pass through a scanning system and a dichroic mirror and further reach the objective and focus the scanning beams as a single spot on the specimen. Although, fluorescence emission generated by the specimen scatter in all directions, it is only the fluorescence from the focal plane of the specimen, which returns via the objective and scanning system. Fluorescence from the focal plane is reflected off the dichroic mirror and focused on a detector. In front of the detector is a spatial filter containing an aperture (detection pinhole), which defines the image of the spot in the focal plane of the microscope. Most fluorescence originating from either above or below the plane of focus of the specimen does not pass through the aperture of the spatial filter and, as a result, little out-of-focus light reaches the detector. Thus the spatial filter not only provides continuous access to the detector for in-focus light, but also effectively suppresses light from out of confocal planes. To form a two-dimensional image, the laser beam scans the specimen horizontally and vertically, and the induced fluorescent light is detected, multiplied and converted into a video signal for display on a computer screen (fig. 1).

Principle of Confocal Laser Scanning Microscopy

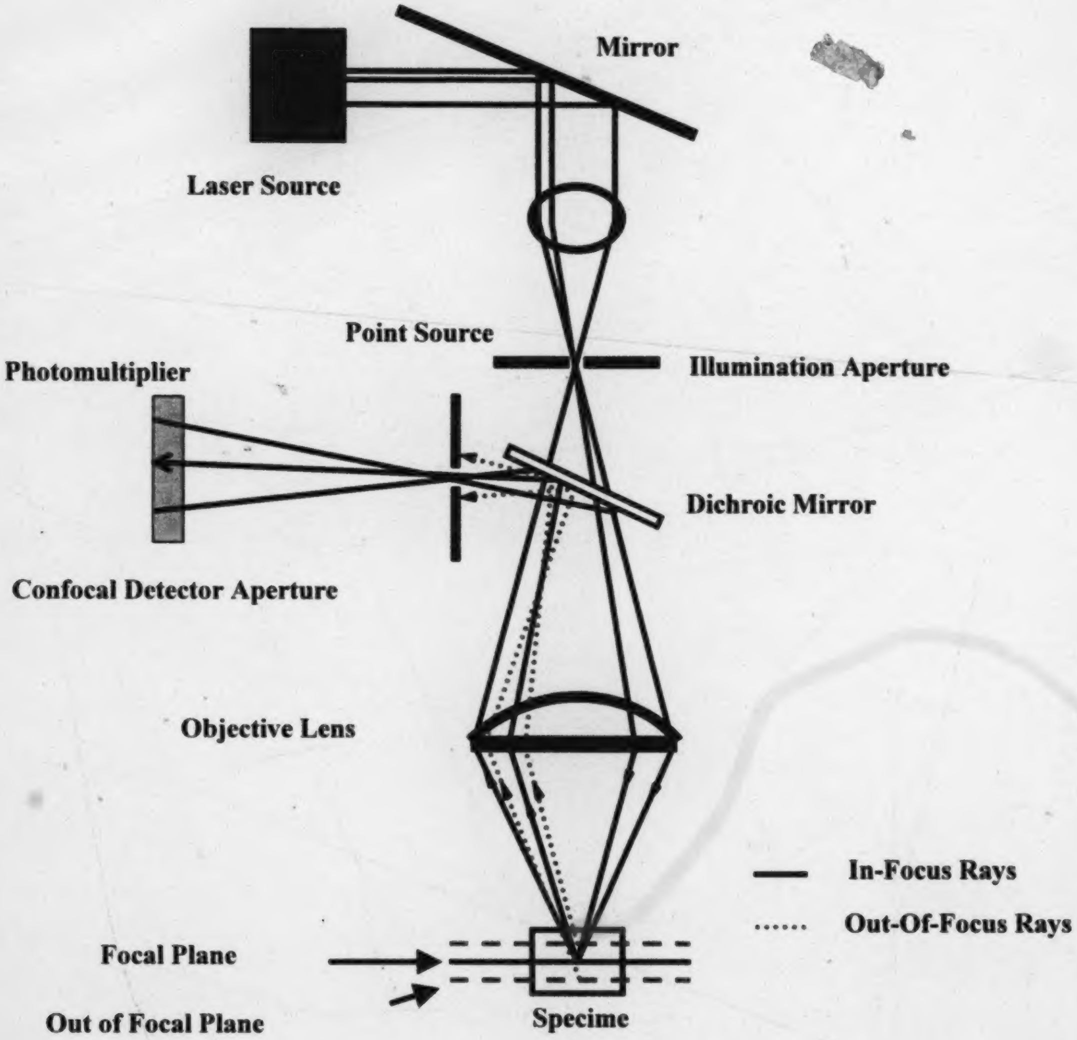

Mirror
Laser Source
Point Source
Photomultiplier
Illumination Aperture
Dichroic Mirror
Confocal Detector Aperture
Objective Lens
In-Focus Rays
Out-Of-Focus Rays
Focal Plane
Out of Focal Plane
Specime

Figure 1. A schematic diagram demonstrating the principle of confocal laser scanning microscopy. Excitation of the fluorochrome-labeled specimen is achieved by the passage of light emitted at the excitation maximum appropriate for the fluorochrome through an illumination aperture. This light passes through a dichroic mirror and is focused on the specimen through the objective lens. Fluorochrome excitation leads to the emission of a longer wavelength light (fluorescence), which returns via the objective and reflects off the dichroic mirror through the imaging aperture (the detection pinhole), and is detected by a photomultiplier tube. Light from above and below the plane of focus is blocked by the pinhole. Thus, out-of-focus light is virtually eliminated from the final confocal image.

Immunofluorescent labeling

Immunofluorescent labeling can be considered as the demonstration and visualization of antigens of interest in tissue sections, cytospins, smears, or cells by the use of specific antigen-antibody interactions. The latter culminate in the attachment of a fluorescent marker to the antigen in question. The aim of immunofluorescent labeling is therefore, to achieve reproducible and consistent demonstrations of specific antigens with a minimum of background staining while at the same time, and ideally, preserving the integrity of tissue or cell architecture (14,15).

Multiple immunofluorescent labeling techniques allow the co-localization and comparison of several cellular components, organelles and mediators, over time. Using a combination of double-immunofluorescent staining and confocal laser scanning microscopy, it is possible to trace the intracellular movement of mediators over time. In double-immunofluorescent staining two different fluorochromes with distinct excitation and emission wavelength, are used to label two components of interest. Images of each labeled component are obtained at various time points and combined. In combined images, color distribution and composition are explanatory of the relative localization of the two-immunostained components of interest. This method could be further extended to study three mediators by employing triple-immunofluorescent staining using three different fluorochromes (15).

Two different immunofluorescent labeling methods, direct and two-step indirect staining are currently used to localize antigens, based on parameters such as type of specimen, degree of sensitivity required, and cost. In the direct technique a fluorescent-labeled antibody reacts directly with the antigen of interest. Direct immunofluorescent labeling is widely used since it utilizes only one antibody and can be completed rapidly. In the two-step indirect method, an unlabelled primary (first layer) antibody is visualized by adding a labeled secondary (second layer) antibody directed against the immunoglobulin of the animal species in which the primary antibody has been raised. This method is more sensitive than the direct method since several fluorescent-labeled antibodies may react with different antigenic sites on the primary antibody. This technique is also more versatile than the direct method as the same fluorescent-labeled secondary antibody can be used with a variety of primary antibodies raised from the same animal species (16)(fig. 2).

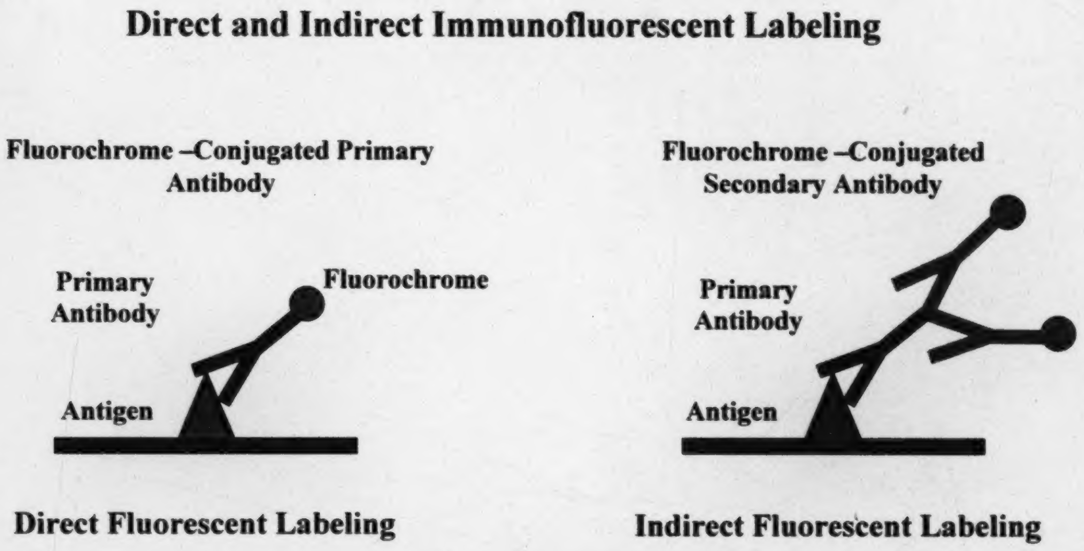

Direct and Indirect Immunofluorescent Labeling
Fluorochrome –Conjugated Primary Antibody
Primary Antibody
Fluorochrome
Antigen
Direct Fluorescent Labeling
Fluorochrome –Conjugated Secondary Antibody
Primary Antibody
Antigen
Indirect Fluorescent Labeling

Figure 2. Direct and indirect immunofluorescent labeling. In the direct method, a fluorochrome-conjugated primary antibody is used to detect and visualize the antigen of interest. In the indirect method, a fluorochrome-conjugated secondary antibody binds to and detects a primary antibody, which is specific for the antigen of interest.

Here we describe confocal laser scanning microscopy and double-immunofluorescent staining methodology employed in intracellular detection, colocalization and tracing of two human eosinophil-derived cytokines, namely interleukin-6 (IL-6) and chemokine RANTES with a well characterized crystalloid granule marker, major basic protein (MBP) in purified peripheral blood eosinophils of asthmatic patients (fig. 3)(17,18).

Materials

Reagents

The following buffers were prepared as 10× stocks and stored at 4°C for many months. All general chemicals were of the highest quality available from commercial sources (Sigma-Aldrich Canada, Mississauga, ON; BDH Inc, Toronto, ON; Fisher Scientific, Fair Lawn, NJ; and ICN Biomedicals Inc, Aurora, OH).

1. Phosphate-buffered saline (PBS) 10× stock solution: 80 g/L NaCl, 2 g/L KCl, 21.71 g/L $Na_2HPO_4.7H_2O$, 2 g/L KH_2PO_4, pH 7.2-7.4. Filtered through 0.22 μm before storage. Final concentration of 1× solution: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4. pH of 1× solution was adjusted to 7.2-7.4 if necessary.
2. Tris-buffered saline (TBS) 10× stock solution: 60.7 g/L Tris-HCl, 87.66 g/L NaCl, pH 7.6. Filtered through 0.22 μm before storage. Final concentration of 1× solution: 100 mM Tris, 150 mM NaCl. pH of 1× solution was adjusted to 7.4 if necessary.
3. RPMI-1640 (BioWhittaker, Walkersville, MD).
4. Paraformaldehyde (2%) (Fisher Scientific) in PBS was prepared just before use. To prepare 2% paraformaldehyde 5 g paraformaldehyde powder was added to 250 mL 1×

PBS, and heated to 55-60°C while stirring on a heating platform until fully dissolved. After cooling the solution to room temperature, it was filtered through 0.22 μm. (Caution: Paraformaldehyde solutions should be prepared in a fume hood as these release toxic formaldehyde gas upon overheating. In addition, since formaldehyde solutions are flammable, they should not be overheated).

5.Bovine serum albumin (ICN Biomedicals, Inc., Aurora, OH).

6.Fetal calf serum (Gibco BRL Life Technologies, Grand Island, NY).

7.Antibleaching solution. 250 μl 10× TBS, 1.87 g glycerol, and 10 mg *n*-propyl gallate (Sigma-Aldrich) were added to a graduated test tube, and made up to 2.5 ml with double-distilled water. Dissolved by vortexing.

8.Dextran (~110,000 Da, Fluka BioChemika, Buchs, Switzerland).

9.Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden)

Anti-CD3, CD14, and CD16-coated immunomagnetic beads (MACS beads; Miltenyi Biotec, Bergisch-Gladbach, Germany).

10. Mouse monoclonal (IgG1) anti-human MBP antibody (BMK-13, generated in-house).

11. Mouse monoclonal (IgG2) anti-human IL-6 antibody (Immunotech, Westbrook, ME).

12. Mouse monoclonal (IgG2) anti-human RANTES antibody (Molecular Probes, Eugene, OR).

13. Goat anti-mouse IgG2 antibody (Molecular Probes).

14. Rhodamine Red-labeled goat polyclonal anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA).

15. BODIPY FL-conjugated goat polyclonal anti-mouse IgG antibody (Molecular Probes).

Apparatus

1. Cytospin 2 centrifuge (Shandon Ltd, Astmoor, Runcorn, UK) for slide preparation.
2. Leica confocal laser scanning microscope system (Leica Lasertechnik GmbH, Heidelberg, Germany), equipped with a krypton/argon laser to allow simultaneous scanning of up to three excitation wavelengths (488, 568 and 647 nm), in order to acquire multiple images from a single pass and reduce bleaching of photosensitive fluorochromes.

Methods

Eosinophil Purification

1. Samples of peripheral blood (50-100 ml), from atopic subjects exhibiting a mild eosinophilia (>5%) not receiving oral corticosteroids, were collected in heparinized tubes.
2. 10 ml 5% dextran was added to whole blood and thoroughly mixed in 50 ml syringes before allowing the erythrocytes to settle by gravitation. Erythrocytes were sedimented for 45 min at room temperature. (Dextran acts by promoting rouleaux formation of erythrocytes).
3. The upper phase of the leukocyte-rich plasma was removed and the plasma was layered onto a 15 ml Ficoll-Paque Plus cushion in a sterile 50 ml Falcon conical centrifuge tube. Gradient was centrifuged for 25 min at 1000*g* at room temperature.
4. Excess clarified plasma, mononuclear layer and excess Ficoll (until <1 ml remained above the granulocyte pellet) were removed.

5.Contaminating erythrocytes were removed by hypotonic lysis on ice by adding 2-3 ml sterile H_2O to the granulocyte pellet for a few seconds, followed by rapid addition of 10-15 ml ice-cold RPMI-1640 and centrifugation.

6.Immediately after washing, the cell pellet was resuspended in 300 µl RPMI , and 12 µl anti-CD16-coated immunomagnetic beads for every 50 × 10^7 cells, plus 10 µl anti-CD3 and 10 µl anti-CD14-coated immunomagnetic beads were added to the total cell suspension.

7.The mixture was incubated for 45 min at 4°C. (It is important not to incubate the beads with cells on ice, as this will reduce the binding ability of the antibody-conjugated beads. This procedure will remove contaminating neutrophils, lymphocytes, and monocytes, respectively, by negative selection).

8.The mixture was added to a freshly prepared magnetic column according to the manufacturer's instructions (Miltenyi Biotec) and purified eosinophils were eluted in a sterile Falcon 50 ml conical tube. The resulting eosinophil purity was greater than 97%.

Slide preparation and immunofluorescent labeling

Following is the method description of double labeling of eosinophil cytospins using the indirect labeling approach.

1. Cytospins of purified eosinophils were prepare by spinning 3-5 × 10^4 cells (suspended in 100 µl 20% FCS in RPMI-1640) in a Cytospin 2 centrifuge at 800 rpm for 2 min.

2. Slides were air-dried for at least 1 h at room temperature in order to permeabilize cells (see Note 1). Slides were wrapped individually in foil and stored at -20ºC until needed.
3. Cytospin slides were fixed for 10 min in freshly prepared 2% paraformaldehyde in PBS and rinsed five times in TBS.
4. Slides were blocked by adding 60 µl 3% FCS in TBS and incubating within a humidified container for 30 min at room temperature (see Note 2).
5. For the first labeling step, slides were washed with TBS and incubated for 1 h at room temperature with 60 µl primary antibody, e.g. 1% mouse monoclonal anti-human MBP (i.e., BMK-13) prepared in TBS containing 1% BSA (see Note 3). An isotype control (5 µg/ml mouse monoclonal IgG1) was used in place of this antibody on a separate cytospin slide to provide a negative control for the immunofluorescence.
6. Slides were washed and incubated with 50 µg/ml Rhodamine Red-labeled goat anti-mouse IgG antibody in TBS containing 1% BSA (60 µl) for 1 h at room temperature.
7. Following another washing step, slides were blocked again for 2 h at room temperature using 50 µg/mL goat anti-mouse IgG in TBS containing 1% BSA (60 µl). This blocking step is necessary for preventing cross-reaction of the primary antibody used in step 5 with the secondary antibody used in step 9 of this method.
8. Doublelabeling was carried out by adding 60 µl primary mouse monoclonal anti-human IL-6 or RANTES (5 µg/mL) in TBS containing 1% BSA and incubating for 1 h at room temperature (see Note 3). Another isotype control antibody

incubation step was included in place of this antibody (5 μg/ml mouse monoclonal IgG1) on the same slide used in step 5 as the negative control for doublelabeling.

9. Following another wash, 20 μg/mL BODIPY FL-conjugated goat anti-mouse IgG in TBS containing 1% BSA (60 μl) was added to each slide and incubated for 1 h at room temperature to detect anti-IL-6 or anti-RANTES antibody binding.
10. 10-μl antibleaching reagent was added to each slide prior to coverslip application. Coverslips were sealed using nail polish.

Confocal laser scanning microscopy

The immunofluorescent staining of eosinophils was examined using a Leica CLSM equipped with a krypton/argon laser, which excites at three major wavelengths (488, 568 and 647 nm). The highest magnification available with a Plan Apo 100X/1.32 oil immersion objective on this system can be used in order to fully resolve the structure of immunolabeled intracellular organelles. The image acquisition settings were optimized using the required pinhole size, photomultiplier gain, and offset. It is possible to achieve a higher spatial resolution by using the appropriate zoom setting on the computer. Following subtraction of eosinophil autofluorescence and nonspecific binding by the use of the isotype control-labeled cytospin slides, images were processed and saved using the Multi-user Multi-tasking Image Analysis software. Images were stored on the computer and transferred as TIF files to Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA) for further image processing.

Notes

The following notes are critical points to bear in mind when investigating intracellular mediator detection and localization using immunofluorescent staining and CLSM.

1. In our hands, air-drying of eosinophil cytospins has been found to be the optimal permeabilization technique for preserving cellular morphology. Addition of permeabilization agents, such as saponin, at later stages has been found to induce a marked deterioration in cell morphology.
2. Care should be taken to reduce autofluorescence and block nonspecific antibody binding through the use of appropriate blocking conditions. In addition, eosinophil granule proteins are highly positively charged, and therefore are readily stained by anionic fluorochromes, such as fluorescein and FITC unless blocked by expensive and stringent blocking proteins (e.g. human IgG). Thus, the use of anionic fluorochromes in staining eosinophils should be avoided. Instead, antibodies conjugated to neutral fluorochromes, such as BODIPY and Rhodamine Red should be employed for eosinophil immunofluorescence studies. Provided that these neutral fluorochromes are used in these studies, it is possible to use conventional blocking conditions (e.g. FCS, goat serum) with eosinophils (see next chapter)(19).
3. The optimal concentration of primary monoclonal antibody preparations in immunofluorescence staining must be determined by titration. For most antibodies, a good starting concentration will be in the range of 1-50 μg/mL.

Optimization of the antibody label should be achieved by comparison of the antibody label with a suitable isotype control at the same concentration.

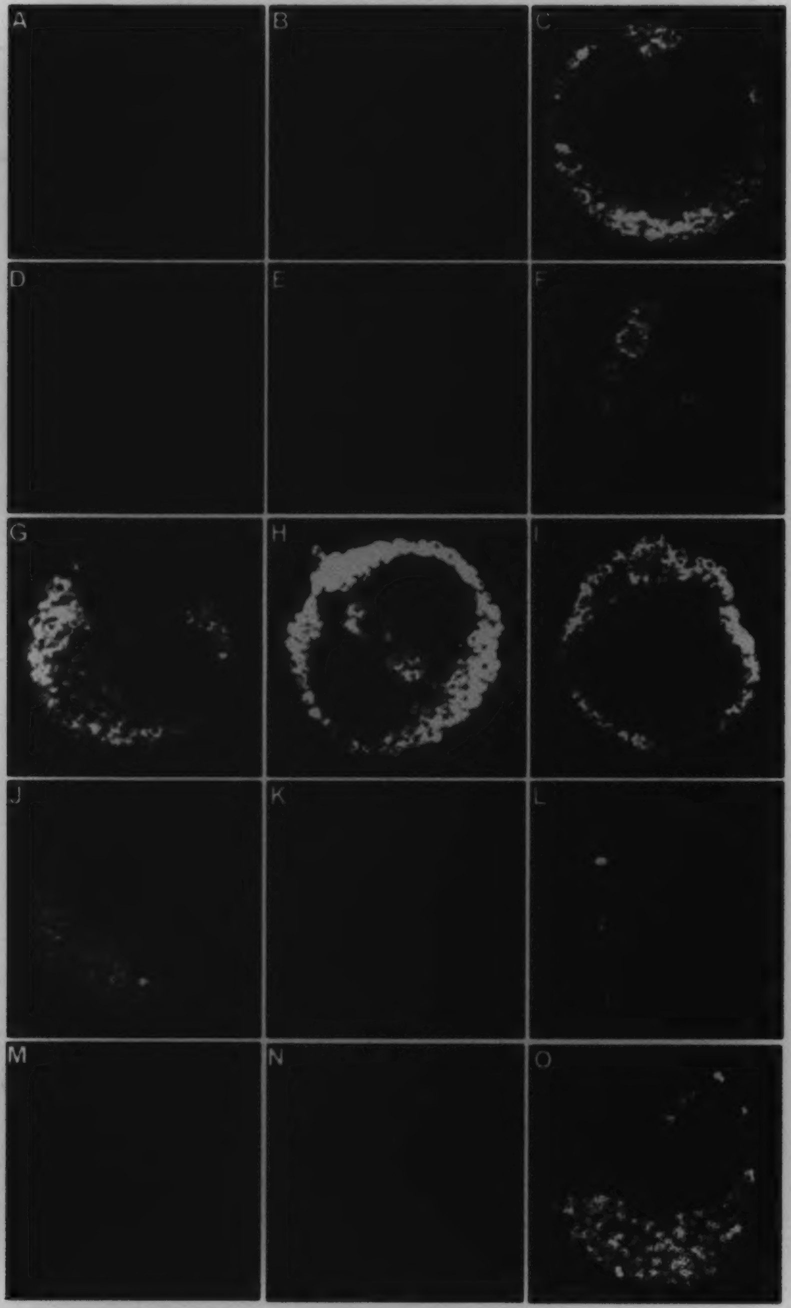


Figure 3. CLSM images of single and double immunofluorescent staining of peripheral blood eosinophils with antibodies for MBP combined with RANTES (A-L), and MBP

combined with IL-6 (M-O). (A-C) are images of unstimulated eosinophils labeled with BODIPY FL indicating RANTES (green color) immunoreactivity (A), Rhodamine Red corresponding to MBP (red color) (B), and combined image (C). (D-F) Higher magnification of eosinophil crystalloid granules showing matrix-associated doughnut-shaped RANTES immunoreactivity (D), surrounding red-labeled cores of MBP immunoreactivity (E), and combined image of the same structure (F). (G-L) Combined images of RANTES and MBP, depicting time course of IFNγ (500 U/mL) stimulation, comparing (G) unstimulated cells with those stimulated for 5 min (H), 10 min (I), 30 min (J), 60 min (K), and 16 h (L). (M-O) Unstimulated eosinophils labeled with BODIPY FL indicating IL-6 immunoreactivity (M), Rhodamine Red corresponding to MBP (N), and combined image (O).

References

1. Lacey, A.J. 1989. Light microscopy in biology: a practical approach. IRL Press, Oxford.
2. Rawlins, D.J. 1992. Light microscopy. Bios Scientific Publishers in association with the Biochemical Society, Oxford.
3. Wischnitzer, S. 1970. Introduction to electron microscopy. Pergamon Press, New York.
4. Paddock, S.W. 1999. Confocal microscopy methods and protocols. Humana Press, Totowa, N.J.
5. Timothy, R.C. and S. Gorden.1996. Confocal scanning optical microscopy and related imaging systems. Academic Press, San Diego.
6. Pawley, J. B. 1990. Handbook of Biological Confocal Microscopy. IMR Press, Madison, Wisconsin.
7. Pawley, J.B., and V. Centonze. 1997. Practical Laser-scanning Confocal Light Microscopy: Obtaining Optimal Performance from Your Instrument. *In* Cell Biology: A Laboratory Handbook. J.E. Celis Editor. Academic Press, New York.
8. Wilson, T., and C.J.R. Sheppared. 1984. Theory and practice of scanning optical microscopy. *Academic Press, New York.*
9. Pawley, J.B. 1997. Fundamental Limits in Confocal Microscopy. *In* Emerging Tools for Single Cell Analysis: Advances in Optical Measurement Technologies. G. Durack, and J.P. Robinson Editors. John Wiley & Sons, New York.
10. Cogswell, C. J., and K. Carlsson. 1994. Three-dimensional microscopy: image acquisition and processing. SPIE. Bellingham, Washington, USA.

11. Wright, SJ. and G. Schatten. 1991. Confocal fluorescence microscopy and three-dimensional reconstruction. *J. Electron Microsc. Tech.* 18:2-10.

12. Minsky, M.1988. Memoir on inventing the confocal scanning microscope. *Scanning* 10:128-138.

13. Minsky, M. 1957. Microscopy Apparatus. U.S. Patent 3,013,467.

14. Sternberger, L.A. 1986. Immunocytochemistry. New York, Wiley.

15. Wick, G.K.N. 1982. Immunofluorescence technology: selected theoretical and clinical aspects. Elsevier Biomedical Press, New York.

16. Caul, E.O. 1992. Immunofluorescence: antigen detection techniques in diagnostic microbiology. Public Health Laboratory Service, London.

17. Lacy, P., F. Levi-Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J. Velazquez, A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon gamma. *Blood*, 91:2508-2516.

18. Lacy, P., S. Mahmudi-Azer, B. Bablitz, S. Hagen, J. Velazquez, P. Man, and R. Moqbel. 1999. Rapid mobilization and release of intracellularly stored RANTES in response to interferon-γ in human eosinophils. *Blood*, 94:23-31.

19. Mahmudi-Azer, S., P. Lacy, B. Bablitz, and R. Moqbel.1998. Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *J. Immunol. Methods*. 217:113-119.

Appendix II

Inhibition of Nonspecific Binding of Fluorescent-Labelled Antibodies to Human Eosinophils[1]

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[1] Authors: S. Mahmudi-Azer, P. Lacy, B. Bablitz and R. Moqbel. A version of this chapter was published in *J. Immunol. Methods* 1998, 217, 113-9. I carried out all experimental procedures described here and prepared and wrote the manuscript.

Introduction

Eosinophils have been proposed to play a major role in a number of different diseases including asthma, allergic diseases, and helminthic infections. In recent years, a wide range of inflammatory and regulatory roles have been ascribed to these cells, particularly in the pathogenesis of various eosinophil-related diseases with an impact on a considerable proportion of the world population (1). Eosinophilia is a hallmark of allergic reactions, where their accumulation at sites of inflammation leads to the release of toxic products, which significantly contribute to the induction of tissue damage (2).

Eosinophils have been shown to synthesize, store, and secrete a wide range of inflammatory mediators, including numerous cytokines and chemokines, with the potential to contribute to the regulation and effector function of these cells (3-11). There is a growing requirement for the development of specialised methods to accurately detect eosinophil products, especially by immunofluorescent staining, in asthma and related allergic conditions. Among the available fluorochromes used in immunofluorescence, fluorescein isothiocyanate (FITC) is the most commonly used conjugate for detection, quantification, and localization of cytokines and other proteins in immune cells. However, it is important to bear in mind that FITC is negatively charged, and will bind positively charged molecules in immunofluorescent staining. This is particularly evident in eosinophils, which possess densely packed cationic proteins in their unique crystalloid granules. Indeed, FITC on its own has been used as an eosinophil-specific stain for immunofluorescence (12-14). Binding by fluorescein and its related compounds has been identified as a major source of false positive results in immunohistochemical analyses, and consequently fluorescein-related molecules has had limited use in reports of eosinophil immunolabelling (15,16). Eosinophil crystalloid granules contain four cationic proteins, namely: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP), which account for the bulk of the granule proteins (17,18). Thus, these arginine-rich granule proteins are responsible for nonspecific binding of FITC-conjugated antibodies.

Although, several methods have been reported to improve the specificity of immunofluorescent techniques in eosinophils, this problem has not yet been fully resolved (19-22). In many cases, these techniques were primarily concerned with reducing autofluorescence in eosinophils, rather than optimisation of immunofluorescence. However, modern techniques such as confocal laser scanning microscopy (CLSM) permit us to screen out eosinophil-derived autofluorescence. In this study, we propose that nonspecific binding by FITC-conjugated antibodies can be eliminated by increasing the potency of blocking reagents. Secondly, by substituting the ionic FITC for a neutral fluorochrome, such as BODIPY FL, the high background caused by charge-based interaction of fluorochromes with eosinophil granule proteins can be reduced. To evaluate these approaches, we have used FITC-conjugated rat monoclonal anti-human interleukin-6 (IL-6) and mouse monoclonal anti-human IL-6 antibody detected by secondary BODIPY FL-conjugated goat anti-mouse antibody. These conjugates were used to detect IL-6 stored within peripheral blood eosinophils purified from asthmatic individuals. We examined immunofluorescence in cytospins of eosinophils by CLSM. In order to test a range of blocking conditions with isotype control and FITC-labelled secondary antibody, we used normal human IgG, fetal calf serum (FCS), bovine serum albumin (BSA), and normal human, goat and horse sera at concentrations ranging between 1-10%. We also investigated BODIPY FL conjugates with a view to eliminating background binding by charged-based interaction of ionic fluorochromes.

Materials and methods

Eosinophil Purification

Samples of peripheral blood (50 ml), obtained from asthmatic subjects who had given their informed consent, were collected in heparin-containing tubes. Erythrocytes were sedimented for 45 min at room temperature with 10 ml 5% dextran (100,000-200,000 kDa; Sigma, Oakville, Ontario, Canada). The upper phase of the leukocyte-rich plasma was then layered onto a 15 ml Ficoll gradient and centrifuged for 25 min at 1000*g*. After removal of excess plasma, mononuclear layer, and Ficoll, the resulting granulocyte pellet was resuspended in 2 ml RPMI-1640 (BioWhittaker, Walkersville, MD, USA). Contaminating erythrocytes were removed by hypotonic lysis on ice in 2-3 ml sterile H_2O for a few seconds. The resultant pellet (containing approximately 5×10^7 cells) was incubated with a mixture of 12 µl anti-CD16, 10 µl anti-CD3 and 10 µl anti-CD14-coated immunomagnetic beads (MACS beads; Miltenyi-Biotec, Bergisch-Gladbach, Germany) for 45 min at 4°C to remove contaminating neutrophils, lymphocytes, and monocytes by negative selection on a magnetic column. The resulting eosinophil purity was usually greater than 99%.

Slide preparations and immunofluorescent labelling

Cytospins of eosinophils were prepared by spinning 3×10^4 cells (suspended in 100 µl 20% FCS in RPMI-1640) in a Cytospin 2 centrifuge (Shandon, UK) at 800 rpm for 2 min. Slides were then foil-wrapped and stored at -20°C until used.

The procedure for fixation and permeabilisation of eosinophils was optimised by using different fixatives and permeabilisation reagents for various periods of time. We used 1, 2, and 4% paraformaldehyde in PBS for 5, 7, and 10 min; ethanol at 30, 45, and 60 sec; 1:1 methanol:acetone for 30, 45, 60, and 120 sec. We also permeabilised cytospins following each fixation condition using

1, 2, and 3% saponin in PBS; 1, 2, and 3% trypsin in PBS; and 1% saponin with 0.1% sodium azide in PBS for 10, 30, and 45 min. Fixing eosinophils with 2% paraformaldehyde for 10 min (in the absence of a permeabilisation step, which usually caused a marked deterioration in cell morphology) was found to be optimal, and thus used for preserving morphology and antigenicity of the preparations.

For experimental analysis, slides of eosinophils were fixed for 10 min in 2% paraformaldehyde in PBS and washed five times in Tris-buffered saline (TBS, pH 7.6). Following fixation, slides were blocked using 1, 2, 5, and 10% (10, 20, 50, and 100 mg/ml) human IgG (Sigma, Reagent Grade I-4506), FCS (Gibco BRL Life Technologies, Grand Island, NY, USA), BSA (ICN Biomedicals, Inc., Aurora, OH, USA), normal human, goat and horse sera (Gibco BRL Life Technologies). Each blocking solution was tested for its efficacy by incubating with eosinophil cytospins for 30, 45, 60, and 120 min at room temperature. After a second washing step, slides were incubated with 5 μg/ml FITC-conjugated monoclonal rat anti-human IL-6 (Pharmingen, San Diego, CA, USA) for 60 min at room temperature. The same concentration of FITC-conjugated rat monoclonal IgG2a (Pharmingen) was used as isotype control. In a parallel experiment, cytospins were incubated with 2 μg/ml mouse monoclonal anti-human IL-6 antibody (Immunotech, Westbrook, Maine, USA) for 60 min at room temperature, and binding of mouse anti-human IL-6 was detected by incubating slides with 20 µg/ml BODIPY FL-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, Oregon, USA) for 60 min at room temperature. After the final wash, 10 µl of antibleaching agent (0.4% *n*-propyl gallate [Sigma] in 3:1 glycerol: TBS) (23) was used before coverslip application.

Confocal laser scanning microscopy

Immunofluorescent staining of eosinophils was examined using a Leica confocal laser scanning microscope (Heidelberg, Germany), equipped with a krypton/argon laser which excites at three major wavelengths (488, 568 and 647 nm). Magnification of specimens was achieved with a Plan Apo objective 100X/1.32 oil immersion. Image acquisition was optimized using the required pinhole setting, photomultiplier gain, and offset. Higher spatial resolution was achieved by using the appropriate zoom on the computer. Eosinophil autofluorescence was subtracted and images were processed and saved using the Multi-user Multi-tasking Image Analysis software. The latter was developed by Leica Lasertechnik GmbH (Heidelberg, Germany) and run on the Motorola 68030 CPU workstation using an OS9 operating system. Images were further analyzed and cropped by Adobe Photoshop 4.0 software (Adobe Systems, Inc., Mountain View, CA, USA).

Results

Effect of normal human IgG on nonspecific binding of FITC-conjugated antibody to eosinophils

Purified peripheral blood eosinophils were immunostained using FITC-conjugated monoclonal rat anti-human IL-6 under different blocking conditions (normal human IgG, FCS, BSA, normal human, goat and horse sera). Blocking reagents were applied at 1, 2, 5, and 10% across 30, 45, 60, and 120 min at room temperature. Among these reagents, only 2% (20 mg/ml) normal human IgG significantly decreased nonspecific binding of primary labelled FITC-conjugated rat monoclonal anti-human IL-6 antibody to eosinophils (Table 1, Fig. 1). The use of a mouse monoclonal anti-human IL-6 antibody detected with a FITC-conjugated secondary antibody generated similar results to the negative control. The effect of increasing concentration of these blocking reagents on background staining is summarized in Table 1.

Blocking Reagent (60 min incubation)	**Concentration**			
	1%	**2%**	**5%**	**10%**
FCS	+	+	+	+
BSA	+	+	+	+
Normal Human Serum	+	+	+	+
Horse Serum	+	+	+	+
Goat Serum	+	+	+	+
Normal Human IgG	+/-	-	+/-	+/-

Table 1. Effects of increasing concentrations of blocking reagents on nonspecific binding of isotype control antibody followed by secondary FITC-conjugated antibodies to eosinophils. The symbols are as follows: (-) reduced nonspecific binding (negligible background), (+) presence of nonspecific binding (high background), and (+/-), weak nonspecific binding. Among the reagents used for this study, no detectable difference was observed at concentrations between 1-10%, except for human IgG which showed maximal blocking efficiency at 2%.

In contrast to normal human IgG, which demonstrated the highest blocking efficiency at 2%, none of the other blocking reagents exhibited detectable effects on nonspecific binding at the concentrations used. Longer incubation times with these blocking reagents did not eliminate background fluorescence (Table 2, for human IgG; data not shown for other blocking reagents). However, normal human IgG was optimal at blocking after 60 min of incubation (Table 2). In addition, a Texas Red-conjugated antibody used to detect antibody binding to MBP in other sets of experiments was found to require human IgG for blocking to prevent charge interaction of this ionic fluorochrome with eosinophil granule proteins (data not shown).

Blocking Reagent	Incubation Time (min)			
	30	45	60	120
Normal Human IgG	+/-	+/-	-	+/-

Table 2. Normal human IgG (2%) showed optimal efficiency of blocking nonspecific binding of FITC-conjugated antibodies to eosinophils after 60 min incubation. Symbols are as described in the legend for Table 1. Incubation of human IgG with eosinophils for 120 min slightly increased nonspecific binding.

A comparison of BODIPY FL and FITC for immunofluorescent staining of eosinophils

Cytospins of purified peripheral blood eosinophils were immunostained using mouse monoclonal anti-human IL-6 antibody. Bound anti-human IL-6 was detected with the use of BODIPY FL-conjugated goat anti-mouse antibody (Fig. 1). A conventional blocking reagent (2% BSA) was sufficient to reduce background staining using BODIPY FL-conjugated antibody. Comparison of images from eosinophils immunostained with FITC- and BODIPY FL-conjugated antibodies (Fig. 1) suggest that these antibodies were similarly able to detect IL-6 provided that sufficient blocking was achieved for FITC-conjugated antibody. They also show similar immunoreactivity corresponding to intracellular localization and distribution of IL-6 within eosinophils. Taken together, we found that FITC-conjugated antibodies employed in eosinophil immunostaining required stronger blocking conditions than those used conventionally. However, BODIPY FL-conjugated antibody exhibited very low nonspecific background and did not require the blocking conditions used with FITC. These findings were confirmed using a mouse monoclonal anti-RANTES antibody detected with FITC- and BODIPY FL-conjugated antibodies. Thus, BODIPY FL-conjugated antibodies appear to be superior to FITC in detecting intracellular proteins in cytospins of human peripheral blood eosinophils.

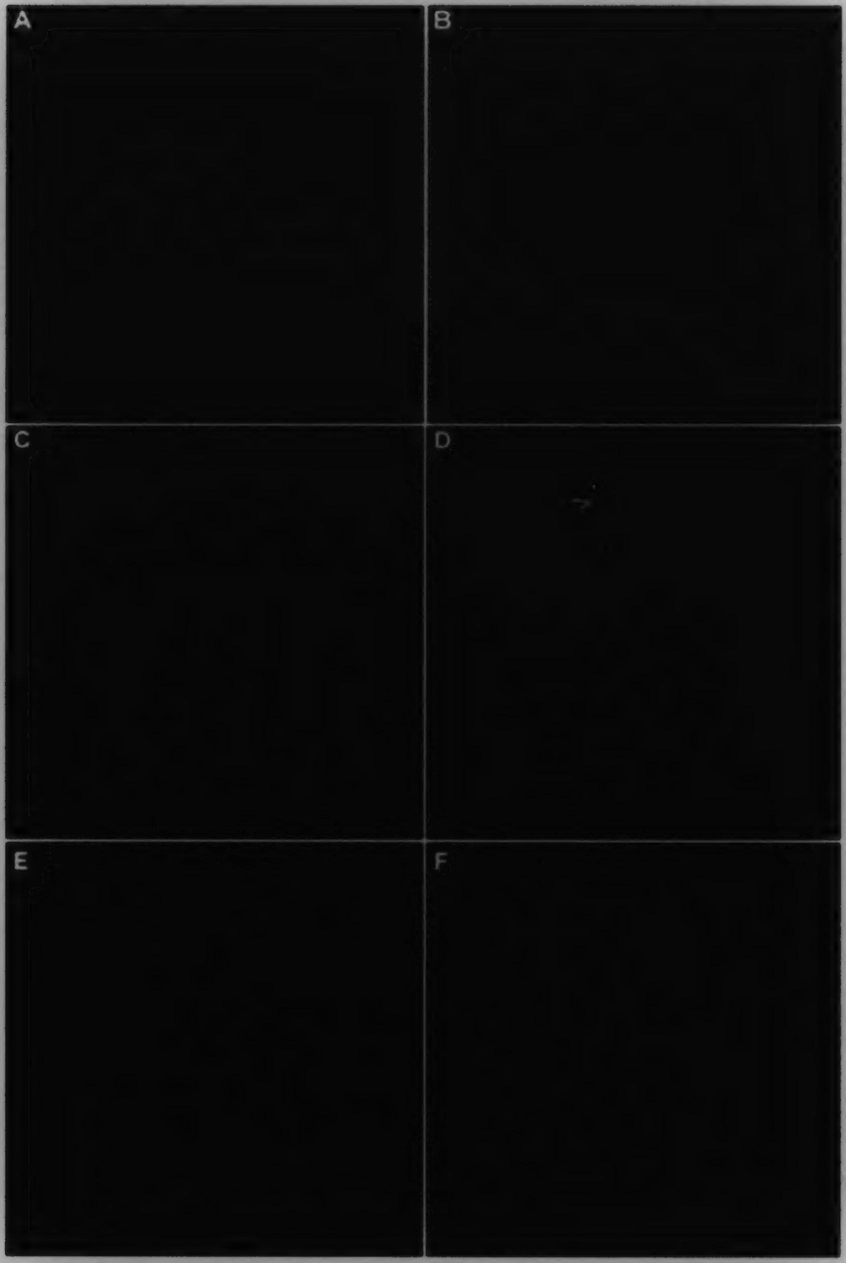
A
B
C
D
E
F

Figure 1. Confocal laser scanning microscopy images of immunofluorescent labelling of IL-6 in cytospins of human peripheral blood eosinophils. Cytospins of eosinophils were labelled with FITC-conjugated rat monoclonal isotype control antibody following incubation for 1 h with a conventional blocking reagent (2% BSA) (A), or 2% (20 mg/ml) human IgG (B) (original magnification 63X). IL-6 immunoreactivity was detected using FITC-conjugated anti-IL-6 blocked with 2% human IgG (D), while negative control with isotype antibody showed negligible nonspecific staining (C). Similarly, IL-6 immunoreactivity could be detected with BODIPY FL-conjugated antibody to anti-IL-6 (F) using conventional blocking conditions (2% BSA), which could be blocked using an isotype control (E). For (C-F), original magnification 100X.

Discussion

In this report we describe two different methods for decreasing nonspecific binding of fluorochrome-conjugated antibodies to eosinophils. Current methods used to detect and localize intracellular products in eosinophils rely on immunocytochemical detection procedures optimised for other inflammatory and immune cell types. However, these methods suffer serious shortcomings when applied to preparations of eosinophils.

We and others have employed FITC-conjugated antibodies for intracellular localisation of eosinophil proteins (22,11). In our studies, we have established that nonspecific binding is eliminated by human IgG (2%). Human IgG, but not other blocking reagents, may prevent association of fluorochrome conjugates to eosinophil proteins by steric hindrance following binding to eosinophil granule proteins. This approach will help in obtaining more accurate visualization of proteins of interest in these and similar studies.

FITC-conjugated antibodies are widely used to detect intracellular proteins in various cell types. In addition to its relatively high absorptivity, excellent fluorescence quantum yield, and good water solubility, FITC has an excitation maximum that closely matches the 488 nm spectral line of the argon-ion laser, making it the fluorochrome of choice for CLSM and flow cytometric applications (24). However, FITC use in immunofluorescent studies has a number of drawbacks, including a comparatively high rate of photobleaching, pH-sensitive fluorescence (significantly reduced below pH 7), and nonspecific binding to positively charged molecules (25). In contrast, BODIPY dyes are insensitive to pH, exhibit greater photostability than fluorescein in some conditions, and lack ionic charge. This suggests that BODIPY may be a better fluorochrome for conjugation in studies of immunofluorescence in eosinophils.

Comparison of the blocking conditions required by FITC- and BODIPY FL-conjugated antibodies in detecting IL-6 suggested that FITC-conjugated antibodies require much stronger blocking conditions in order to prevent nonspecific binding. Similar results were obtained using either rat or mouse monoclonal antibodies detected by FITC conjugates. We also observed similar nonspecific binding by another negatively charged fluorochrome used as a conjugate for secondary antibody, namely Texas Red (data not shown), suggesting that human IgG is a suitable blocking reagent for most ionic fluorochromes used in immunofluorescent labelling of eosinophils. All findings reported in this paper using antibodies to IL-6 were reproduced in parallel experiments employing antibodies specific for RANTES. We have concluded that since BODIPY FL-conjugated antibodies do not require stringent blocking reagents, they present a preferable option for use in immunofluorescent staining of eosinophil proteins.

These findings may have implications for future studies involving immunofluorescent staining of intracellular proteins in eosinophil cytospins and eosinophil-containing biopsy sections. The difficulty in blocking nonspecific antibody binding to eosinophils during colour substrate reactions (e.g., during APAAP staining) may also be eliminated by the use of blocking conditions as outlined above, although it is important to remember that ionic fluorochromes pose a special challenge due to additional staining caused by the detection molecule. We anticipate that these findings will provide a significant improvement in fluorescent immunostaining of eosinophils.

References

1. Wardlaw, A.J., R. Moqbel, and A.B. Kay. 1995. Eosinophils: biology and role in disease. *Adv. Immunol.* 60, 151-266.
2. Moqbel, R. 1996. Synthesis and storage of regulatory cytokines in human eosinophils. *Adv. Exp. Med. Biol.* 409, 287-294.
3. Dubucquoi, S.P., P. Desreumaux, A. Janin, O. Klein, M. Goldman, J. Tavernier, A. Capron, and M. Capron. 1994. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *J. Exp. Med.* 179, 703-708.
4. Moqbel, R., F. Levi-Schaffer, and A.B. Kay. 1994. Cytokine generation by eosinophils. *J. Allergy Clin. Immunol.* 94, 1183-1188.
5. Weller, P.F. 1994. Eosinophils: structure and functions. *Curr. Op. Immunol.* 6, 85-90.
6. Levi-Schaffer, F., P. Lacy, N.J. Severs, T.M. Newman, J. North, B. Gomperts, A.B. Kay, and R. Moqbel. 1995. Association of granulocyte/macrophage colony-stimulating factor with the crystalloid granule of human eosinophils. *Blood* 85, 2579-2586.
7. Moqbel, R., S. Ying, J. Barkans, T.M. Newman, P. Kimmitt, M. Wakelin, L. Taborda-Barata, Q. Meng, C.J. Corrigan, S.R. Durham, and A.B. Kay. 1995.

Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *J. Immunol.* 155, 4939-4947.

8. Egesten, A., J. Calafat, E.F. Knol, H. Janssen, and T.M. Walz. 1996. Subcellular localization of transforming growth factor-alpha in human eosinophil granulocytes. *Blood* 87, 3910-3918.
9. Kita, H. 1996. The eosinophil: a cytokine-producing cell? *J. Allergy Clin. Immunol.* 97, 889-892.
10. Levi-Schaffer, F., J. Barkans, T.M. Newman, S. Ying, M. Wakelin, R. Hohenstein, V. Barak, P. Lacy, A.B. Kay, and R. Moqbel. 1996. Identification of interleukin-2 in human peripheral blood eosinophils. *Immunology* 8, 155-161.
11. Lacy, P., F. Levi-Schaffer, S. Mahmudi-Azer, B. Bablitz, S.C. Hagen, J. Velazquez, A.B. Kay, and R. Moqbel. 1998. Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon-γ. *Blood* 91, 2508-2516.
12. Nairn, R.C. 1969. Immunological Tracing: General Considerations. In *Fluorescent Protein Tracing,* 3rd ed. E. & S. Livingstone, Edinburgh, p. 136.
13. Filley, W.V., G.M. Kephart, K.E. Holley, and G.J. Gleich. 1982. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* 2, 11-16.

14. Nonaka, M., R. Nonaka, and K. Woolley. 1995. Distinct immunohistochemical localization of IL-4 in human inflamed airway tissues. *J. Immunol.* 155, 3234-3244.

15. Floyd, K., P.F. Suter, and H. Lutz. 1983. Granules of blood eosinophils are stained directly by anti-immunoglobulin fluorescein isothiocyanate conjugates. *Am. J. Vet. Res.* 44, 2060-2063.

16. Detlefs, R.L., I. J. Frieden, T.G. Berger, and D. Westrom. 1987. Eosinophil fluorescence: a cause of false positive slide tests for *Herpes simplex* virus. *Ped. Dermatol.* 4, 129-133.

17. Ackerman, S.J., D.A. Loegering, P. Venge, I. Olsson, J.B. Harley, A.S. Fauci, and G.J. Gleich. 1983. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* 131, 2977-2982.

18. Gleich, G.J., C.R. Adolphson, and K.M. Lieferman. 1993. The biology of the eosinophilic leukocyte. Ann. Rev. Med. 44, 85-101.

19. Beutner, E.H., M.R. Sepulveda, and E.V. Barnet. 1968. Quantitative studies of immunofluorescent staining. Relationships of characteristics of unabsorbed antihuman IgG conjugates to their specific and nonspecific staining properties in an indirect test for antinuclear factors. *Bull World Health Organ* 39, 587-606.

20. Nairn, R.C., F. Herzog, H.A. Ward, and W.G. De Boer. 1969. Microphotometry in immunofluorescence. *Clin. Exp. Immunol.* 4, 697-705.

21. Johnston, N.W., and J. Bienenstock. 1974. Abolition of nonspecific fluorescent staining of eosinophils. *J.Immunol. Methods* 4, 189-194.

22. Krug, N., A.M. Thurau, P. Lackie, J. Baier, G. Schultze-Werninghaus, C.H. Rieger, and U. Schauer. 1996. A flow cytometric method for the detection of intracellular basic proteins in unseparated peripheral blood and bone marrow eosinophils. *J. Immunol. Methods* 190, 245-254.

23. Krenik, K.D., G.M. Kephart, K.P. Offord, S.L. Dunnette, G.J. Gleich. 1989. Comparison of antifading agent used in immunofluorescence. *J. Immunol. Methods.* 117, 91-97.

24. Wells, S., and I. Johnson. 1994. Three-Dimensional Confocal Microscopy. In *Investigation of Biological Specimens.* J.K. Steven, L.R. Mills, and J.E. Trogadis eds. Academic Press, San Diego, pp. 101.

25. Robenson, J.L., and R.D. Tilton. 1995. Effect of fluorescence quenching on fluorescence recovery after photobleaching measurements. *Biophys. Journal* 68, 2145-2155.

Appendix III

Curriculum Vitae

Curriculum Vitae

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Current Education: 1996-Present: Ph. D. candidate, Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB

Supervisor: Dr. Redwan Moqbel

Title of Study: Expression of CD63 in resting vs. activated human peripheral blood eosinophils.

Completion Date: Spring of year 2001

Courses taken during the graduate program between 1996 and present

Year	Course	Course Title	Grade
1997/1998	Immun 451	Advanced Immunology	7
1997/1998	Cell 495	Cell Biology	9
1997/1998	Med 571	Eosinophil Studies	9
1996/1998	Med 671	Seminar Course	Pass
1998/1999	Physl 512	Physiology of the Respiratory System	8

2000/2001	Med 573	Clinical Medicine	9

Others

1996	Radiation Protection Course

Previous Education

- Master of Science, Major in Genetics, Tehran University, Iran (1990)
- Bachelor of Science, Major in Genetics, Chamran University, Ahwaz, Iran (1988)

Research Experience

- Leukocytes separation from peripheral blood of asthmatic and control subjects
- Purification of eosinophils using negative immunomagnetic selection techniques
- Kinetics of *in vitro* stimulation of eosinophils using various agonists
- Cytospin preparation
- Immunocytochemistry (APAAP staining)
- Single and double immunofluorescence staining
- Fluorescent and confocal laser scanning microscopy
- Flowcytometry and FACS analysis
- Immunoprecipitation and Western blotting
- Metabolic labeling (pulse chase) and Endo-H treatment
- DNA extraction, purification and sequencing
- PCR analysis
- In-situ RT-PCR

- Plant, harvest, stain, and photograph chromosome cultures and measure chromosome lengths and numbers to determine their structural and numerical abnormalities.

Work History

- Postgraduate student, Pulmonary Research Group, Dept. of Medicine, University of Alberta (1996- Present)
- Student, Calgary Catholic Immigration Society (1995-1996)
- Genetic Counselor, Children's Hospital, Orumiya, Iran (1992-1994)
- Teaching Assistant, Medical Sciences University, Orumiya University, Iran (1991-1994)

Personal Achievements

- Recipient of the "Canadian Society for Immunology 15th Annual Spring Meeting Abstract Award" (2001)
- Recipient of the "Canadian Society for Immunology 15th Annual Spring Meeting First Place Travel Award", (Cedarlane Travel Award) (2001)
- Recipient of the "Alberta Heritage Foundation for Medical Research Studentship Award" (2001)
- Recipient of "Canadian Institutes of Health Research (Medical Research Council of Canada) Gold Prize in the National Health Research Poster Competition (2000)
- Recipient of "Best Presentation Award" at Department of Medicine Research Day, University of Alberta (2000)
- Recipient of the "Alberta Heritage Foundation for Medical Research Studentship Award" (2000)

- Selected by the Alberta Graduate Council to present in media launch (2000)
- Recipient of the "Alberta Lung Association Studentship Award" (1999)
- Recipient of the "Canadian Society for Immunology Travel Award" (1999)
- Recipient of the "Faculty of Medicine and Dentistry 75th Anniversary Studentship Award" (1998-1999)
- Recipient of "Best Abstract Award" at Alberta Respiratory Diseases Symposium (1998)
- Recipient of "Second Best Presentation Award" at Immunology Network Retreat symposium (1998)
- Recipient of the "Best Instructor" National Award (1992)
- Excellent marks throughout academic career, including the highest marks in Embryology, Microbiology, Oncology, Biochemistry, Cellular Immunology, and Evolution
- Fluent in English, Kurdish, Persian, Turkish and Azary
- Published one novel and one book of Poetry
- Played Volleyball for the Iranian National University Team

Volunteer and extracurricular activities

- Active member (since 1996) and chairperson (2000-2001) of Amnesty International, U of A chapter
- Active member and chairperson of International Society for Peace and Human Rights (1998-2001).
- Member of International Secretariat for Peace and Human Rights (2000-2001)

- Member of Kurdish Writers Association (1987-1994)

Conferences Attended

- Canadian Institutes of Health Research National Health Research Conference (June 2000)
- International Eosinophil Symposium (May 2001)
- Annual Alberta Respiratory Disease Symposium (April 2001)
- Annual meeting of Canadian Society for Immunology (April 2001)
- Annual meeting of Immunology Student Network (June 2000)
- Annual meeting of Immunology Student Network (June 1999)
- Annual meeting of Canadian Society for Immunology (March 1999)
- Annual Alberta Respiratory Disease Symposium (October 1998)
- Annual meeting of Immunology Student Network (June 1998)
- Annual meeting of Canadian Society for Immunology (March 1998)
- Annual Alberta Respiratory Disease Symposium (October 1997)
- Annual meeting of Immunology Student Network (September 1997)
- Annual meeting of Microscopical Society of Canada (June 1997)
- Annual meeting of Canadian Society for Immunology (March 1997)

Publications

Peer-reviewed

1. Lacy, P., Levi-Schaffer, F., **Mahmudi-Azer, S.**, Bablitz, B., Hagen, S.C., Velazquez, J., Kay, A.B. and Moqbel, R. (1998) Intracellular localization of interleukin-6 in eosinophils from atopic asthmatics and effects of interferon-γ. *Blood* 91:2508-2516.
2. **Mahmudi-Azer, S.**, Lacy, P., Bablitz, B., and Moqbel, R. (1998) Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *J. Immunol. Methods* 217:113-119.
3. Velazquez, J.R., Lacy, P., **Mahmudi-Azer, S.**, and Moqbel, R. (1999) Effects of interferon-γ on mobilization and release of eosinophil-derived RANTES. *Int. Arch. Allergy Immunol.* 118:447-449.
4. Lacy, P., **Mahmudi-Azer, S.**, Bablitz, B., Hagen, S.C., Velazquez, J.R., Man, S.F.P., and Moqbel R. (1999) Rapid mobilization of intracellularly stored RANTES in response to interferon-γ in human eosinophils. *Blood* 94:23-32.
5. Lacy, P., **Mahmudi-Azer, S.**, Bablitz, B., Gilchrist, M., Fitzharris, P., Cheng, D., Bokoch, G.M., Man, S.F.P. and Moqbel, R. Expression and translocation of Rac2 in eosinophils during superoxide generation. *J. of Immunolo* 98:244-252.
6. Vliagoftis H., Hutson A., **Mahmudi-Azer S.**, Rumsaeg V., Chad O., Moqbel R., and Metcalfe DD. (1999) Mast cells express Connexin on their cytoplasmic membrane. *J. Allergy Clin. Immunol.* 103: 656-662.
7. **Mahmudi-Azer S.**, Velazquez JR., Lacy P., Denburg J., and Moqbel R. (2000) Immunofluorescence analysis of cytokine and granule protein expression during

eosinophil maturation from cord blood-derived CD34$^+$ progenitors. *J. Allergy Clin. Immunol.* 105:1178-1184.

8. Velazquez, J.R., Lacy, P., **Mahmudi-Azer, S.**, Bablitz, B., Milne, C., and Moqbel, R. (2000) Interleukin-4 and RANTES expression in maturing eosinophils derived from human cord blood CD34+ progenitors. *Immunology.* 101:419-425.
9. **Mahmudi-Azer S.**, and Moqbel R. The association of the tetraspanin, CD63, with agonist-induced exocytosis in eosinophils from asthmatic subjects (Submitted).

Abstracts:

1. Moqbel R., Hagen S, Bablitz B., Velazquez J, **Mahmudi-Azer S.**, Lacy P. 1997. Kinetics of physiological release of RANTES from eosinophils obtained from asthmatic subjects. *Abstracts of the Canadian Society for Immunology annual meeting, Lake Louise, Alberta, Canada (March 14-1), (Abstract 98).*
2. Lacy P., **Mahmudi-Azer S.**, Hagen S., Bablitz B., Levi-Schaffer F., Moqbel R. 1997. Interleukin-6: synthesis in eosinophils and storage of the translated product in association with their crystalloid granules. *Abstracts of the Canadian Society for Immunology annual meeting, Lake Louise, Alberta, Canada (March 14-17), (Abstract 99).*
3. Lacy P., Hagen S., Bablitz B., Velazquez J., **Mahmudi-Azer S.**, Moqbel R. 1997. Regulation of physiological release of RANTES from eosinophils obtained from asthmatic patients. *Abstracts of Annual General meeting of INSPIRAPLEX (Respiratory Health Network of Centers of Excellence), Montreal, PQ, Canada (April 24-26).*

4. Lacy, P., Hagen, S., Bablitz, B., Velazquez, J., **Mahmudi-Azer, S.,** Moqbel, R. 1997. Regulation of physiologic release of RANTES from eosinophils obtained from asthmatic patients. *Clin. Invest. Med. (Suppl. to August 1997)* ***20**:3.*
5. Lacy, P., **Mahmudi-Azer, S.,** Hagen, S.C., Bablitz, B., Moqbel, R. 1997. Interleukin-6 localization to the matrix of secretory granules in human asthmatic eosinophils. *Abstracts for the Canadian Resident-Fellow Competition, Vancouver, BC, Canada (September 24-25).*
6. Lacy P., Levi-Schaffer F., **Mahmudi-Azer S.,** Bablitz B., Hagen SC., Velazquez J., Moqbel R. 1997. Effects of interferon-γ on localization of interleukin-6 (IL-6) in peripheral blood eosinophils from atopic asthmatics. *Proceedings of the 4th Annual Alberta Respiratory Diseases Symposium, October, Banff, Alberta, Canada (October 17-19).*
7. Moqbel R., **Mahmudi-Azer S.**, Bablitz B., Velazquez J., Hagen SC., Lacy P.1998. Early effects of interferon-γ on release of RANTES in eosinophils from atopic asthmatics. *Journal of Allergy and Clinical Immunology, 101 (No 1,part 2); S170 (Abstract 703).*
8. Lacy P., **Mahmudi-Azer S.**, Cheng D., Fitzharris P., Bokoch G., Moqbel R. 1998. Rac2 translocation in stimulated eosinophils. *Journal of Allergy and Clinical Immunology, 101 (No 1, part 2); S212 (Abstract 878).*
9. **Mahmudi-Azer S.**, Lacy P., Bablitz B., Moqbel R. 1998. Blocking non-specific binding of fluorescent-labeled antibodies to human eosinophils by using neutral fluorochromes. *Journal of Allergy and Clinical Immunology, 101 (No 1, part 2); S211 (Abstract 877).*

10. Lacy P., **Mahmudi-Azer S.**, Levi-Schaffer F., Hagen SC., Bablitz B., Velazquez J., Moqbel R. 1998. Interferon-γ induces early interleukin-6 (IL-6) mobilization in human asthmatic eosinophils. *FASEB Journal, 12 (Part 1); A592 (Abstract 3437).*
11. Velazquez J., Lacy P., **Mahmudi-Azer S.**, Hagen SC., Bablitz B., Moqbel R.1998. Regulation of RANTES gene expression from asthmatics. *FASEB Journal, 12 (Part 1); A593 (Abstract 3441).*
12. Lacy, P., **Mahmudi-Azer, S.,** Bablitz, B., Fitzharris, P., Bokoch, G., Moqbel, R. 1998. Expression of NADPH oxidase components in eosinophils. *Abstracts for Molecular Mechanisms of Signal Transduction, 33rd meeting of the Society for Leukocyte Biology, San Diego, CA, U.S.A. (August 22-25).*
13. Moqbel R., **Mahmudi-Azer S.**, Bablitz B., Velazquez J., Lacy P. 1998. Effects of interferon-γ on mobilization and release of eosinophil-derived RANTES. *Abstracts for Meeting of the Collegium Internationale Allergologicum, Corfu, Greece, (September, 9-13) (Abstract # 69, p4).*
14. **Mahmudi-Azer S.**, Lacy P, Bablitz B, Moqbel R. 1998. Inhibition of non-specific binding of fluorescent-labeled antibodies to human eosinophils by using different blocking conditions and neutral fluorochromes. *Abstracts for the 5th Annual Alberta Respiratory Diseases Symposium, Banff, Alberta, Canada (October 16-18).*
15. Lacy, P., Bablitz, B., **Mahmudi-Azer, S.,** Gilchrist, M., Man, S.F.P., Moqbel, R. 1998. Expression and activation of Rac2 and NADPH oxidase complex in eosinophils. *Abstracts for the 5th Annual Alberta Respiratory Diseases Symposium, Banff, Alberta, Canada (October 16-18).*

16. **Mahmudi-Azer S.**, Velazquez JR, Lacy P, Moqbel R. 1999. Evidence for differential transport of granule proteins during interleukin-5-dependent differentiation of eosinophil colonies from human CD34+ progenitors. *J. Allergy Clin. Immunol. 103:S112 (Abstract 428).*

17. Lacy P., Bablitz B., **Mahmudi-Azer S.**, Gilchrist M., Fitzharris P., Cheng D. Bokoch GM., Man SFP, Moqbel R. 1999. Superoxide generation from eosinophils involves translocation of NADPH oxidase components. *J. Allergy Clin. Immunol. 103:S111 (Abstract 422).*

18. Velazquez JR., **Mahmudi-Azer S.**, Lacy P., Moqbel R. 1999. Differential expression of cytokines in CD34+-Derived eosinophils cultured in semi-solid and liquid media. *J. Allergy Clin. Immunol. 103:S183 (Abstract 701).*

19. **Mahmudi-Azer S.**, Velazquez JR, Lacy P, Moqbel R. 1999. Intracellular differential transport of granule proteins during interleukin-5-dependent differentiation of eosinophil colonies from human CD34+ progenitors. *Abstracts of the Canadian Society for Immunology annual meeting, Lake Louise (March 5-8).*

20. Lacy, P., **Mahmudi-Azer, S.**, Moqbel, R. 1999. Expression of SNAREs: Regulatory molecules implicated in degranulation in human eosinophils. *Abstracts for the 6th Annual Alberta Respiratory Diseases Symposium, Banff, AB, (October 22-24). Winner of Best Abstract Award.*

21. **Mahmudi-Azer S.**, Moqbel R. 1999. Expression of CD63 in resting and activated human peripheral blood eosinophils. *Proceedings of the 6th Annual Alberta Respiratory Diseases Symposium, Banff, Alberta, Canada (October22-24).*

22. Lacy, P., Johri, A., **Mahmudi-Azer, S.,** Moqbel, R. 2000. Expression of the SNARE isoform SNAP-23 in human eosinophils. *J. Allergy Clin. Immunol. 105:S252 (Abstr. 749).*
23. Lacy, P., Johri, A., **Mahmudi-Azer, S.,** Moqbel, R. 2000. Do eosinophils express the exocytotic fusion protein syntaxin-4? *Am. J. Resp. Crit. Care Med.* 161:A539.
24. **Mahmudi-Azer S.,** Moqbel R. Expression of CD63 in resting and activated human peripheral blood eosinophils. *J. Allergy Clin. Immunol. 105:S252 (Abstr. 764) (March 3-8).*
25. **Mahmudi-Azer S.,** Moqbel R. 2000. CD63 in human peripheral blood eosinophils. *Clin. Invest. Med. Vol 23, No. 3, June.*
26. **Mahmudi-Azer S.,** Moqbel R. 2000. Expression of CD63 in resting and activated human peripheral blood eosinophils. *National Graduate Student Research Poster Competition, Faculty of Medicine, University of Manitoba (June 7-9).*
27. **Mahmudi-Azer S.,** Moqbel R. 2000. Expression of CD63 in resting and activated human peripheral blood eosinophils. *Abstracts for the Immunet Retreat, Camp Nakamun, Edmonton, Alberta, Canada (June 12).*
28. **Mahmudi-Azer S.,** Moqbel R. 2000. Expression of CD63 in resting versus activated human peripheral blood eosinophils. *Abstracts for The Canadian Society for Clinical Investigation, Young Investigators Forum, Edmonton, Alberta, Canada (September 21)(Abstract 24).*
29. **Mahmudi-Azer S.,** Moqbel R. 2001. The Tetraspanin, CD63, is expressed in human eosinophils and may be associated with piecemeal degranulation. *J. Allergy Clin. Immunol. 107:S143 (Abstr. 148) (March 16-21).*

30. Mahmudi-Azer S., Moqbel R. 2001. CD63 association with agonist-induced exocytosis in eosinophils from asthmatic subjects. *15th Annual Spring Meeting of The Canadian Society for Immunologist. Lake Louise, Alberta, Canada (April 6-9).*
31. Mahmudi-Azer S., Moqbel R. 2001. CD63 expression in human peripheral blood eosinophils and its potential association with mediator release. *Abstracts for Alberta Respiratory Diseases Symposium, Kananaskis, Alberta, Canada (26-29 April).*
32. **Mahmudi-Azer S.,** Moqbel R. 2001. Tetraspanin, CD63, is associated with agonist-induced exocytosis in eosinophils from asthmatic subjects. *Abstracts for Department of Medicine Research Day. Edmonton, Alberta, Canada (May 3).*
33. **Mahmudi-Azer S.,** Moqbel R. 2001. Potential association of Tetraspanin, CD63 with agonist-induced piecemeal degranulation in eosinophils of asthmatic individuals. 2001. *Abstracts for First Symposium or the International Eosinophil Society. Banff, Alberta, Canada, (May 4-8).*

Chapters and Reviews

1. **Mahmudi-Azer S.**, Lacy P, Moqbel R. Tracing intracellular mediator storage and mobilization in eosinophils using confocal microscopy and subcellular fractionation. In: Human Airway Inflammation: Sampling Techniques an Analytical Protocols, DF Rogers and LE Donelly (Editors), Human Press Inc.